STUDIES ON SPERMATOGENESIS AND APOPTOSIS IN THE BOVINE

By

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by

Victor Hugo Monterroso Pérez

Esta disertación esta dedicada al Señor Dios todo poderoso, quien creo los cielos y la tierra, Dios de Israel, Jehová de los ejércitos. Dios que me infunde aliento, quien nunca me ha dejado, quien durante el día me ha guiado con su nube y con su luz durante la noche, quien calma la mar durante la tormenta y el que me lleva a puerto seguro. Al Dios que me ha permitido caminar en seco en medio de la mar y en camino donde no lo ha habido, El que ha entregado al enemigo en mis manos, El que ha aderezado meza delante de mi, El que ha entregado ésta victoria en mis manos, El que me ha permitido alcanzar esta meta, a quien pertenece toda gloria y honra, a quien pertenece el conocimiento y la sabiduria, al Dios de mi salvación.

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ABBREVIATIONS

AI	Artificial insemination
ANOVA	Analysis of variance
BA	Brahman x Angus
BSE	Breeding soundness evaluation
B. taurus	Bos taurus
B. indicus	Bos indicus
BW	Body weight
°C	Celsius degrees
Ca	Calcium
CP	Crude protein
CSM	Cottonseed meal
Cu	Copper
DM	Dry matter
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate Buffered Saline
DSP	Daily sperm production
DSPG	Daily sperm production per g
EDTA	Ethylenediaminetetraacetic acid
FSH	Follicle-stimulating hormone
GnRH	Gonadotropin releasing hormone
GSH	Reduced glutathione
hCG	human chorionic gonadotropin
Hsp	Heat shock protein
IL-6	Interleukin-6
IV	Intravenous
IVF	In vitro fertilization
K	Potassium
LH	Luteinizing hormone
LSMeans	Least squares means
MDA	Malondiadldehyde
Mg	Magnesium
MM	microtiterplate-modules
Mw	Molecular weight
N	North
•OH	Hydroxyl radical

P	Phosphorus
PGF1α	Prostaglandin F-1 alpha
ppm	Parts per million
ROS	Reactive oxygen species
SA	Senepol x Angus
SBM	Soybean meal
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SI	Scrotal insulation
TA	Tuli x Angus
TD	Time divisor
TdT	terminal deoxynucleotidil transferase
TMR	Total mixed ration
TNF	Tumor necrosis factor
vs	Versus
v:v	Volume:volume
W	West
w:v	Weight:volume

UNITS

U	Units
IU	International unit
kg	Kilogram
g	Gram
mg	Milligram
µg	Microgram
l	Liter
M	Molar
ml	Milliliter
µl	Microliter
mU	Milliunits
mo	Month
wk	Week
d	Day
h	Hour
min	Minutes

Abstract of Dissertation Presented to the Graduate School
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The main objective was to study the effect of elevated temperatures, gossypol, Vitamin E, and breed differences on spermatogenesis and spermatogenic apoptosis. First, induction and assessment of apoptosis were studied. Experiment (exp.) 1 (Angus, n=11), included; 1) control, 2) E. coli endotoxin, and 3) scrotal insulation (SI). Spermatogenesis (daily sperm production, DSP and DSP/g, DSPG) and apoptosis were assessed at 10 d. Experiment 2, (Angus, An; Senepol, Se; Romosinuano, Ro; n=18) included; 1) control and 2) SI. Assessment was done at 2 d and 4 d. In exp. 1 and 2 treatment had no effect on spermatogenesis and apoptosis.

Second, effects of gossypol and vitamin E on spermatogenesis and apoptosis were tested. Young Holstein bulls (n=24) were supplemented with either; 1) CONT (soybean meal, corn, and vitamin E, 2) GOSS (cotton seed meal, CSM, corn, and vitamin E), and

3) G+VITE (CSM, corn, and vitamin E). Spermatogenesis was less ($P \leq 0.05$) in GOSS than CONT or G+VITE. Gossypol had no effect on apoptosis, although there was an inverse relationship between spermatogenesis and apoptosis ($P<0.05$).

Third, young B. taurus, B. indicus, and crossbred bulls were evaluated during the summer in Florida. In exp. 1 (n=36), An, Ro, Brahman (Br), and Nelore x Brahman bulls were used. Breed had no effect on spermatogenesis, extragonadal sperm reserves (ESR) (caput), and ESR (cauda). However, ESR (corpus) in Br was higher than in An and Ro ($P \leq 0.05$). In exp. 2, crossbred bulls (n=112; Senepol x Angus, SA; Tuli x Angus, TA; Brahman x Angus, BA) were used, and DSP (perhaps DSPG) was less in BA than in SA and TA ($P<0.05$). In exp. 3, mature Brahman (n=2) and miniature Brahman (n=5) bulls were used, and DSP was less in miniature than in Brahman bulls ($P<0.05$). In exp. 2 and 3 breed did not affect apoptosis.

This study failed to observed increases in spermatogenic apoptosis as a result of E. coli endotoxin, SI, gossypol, summer temperatures, and breed. In general, low levels of apoptosis were detected and reasons for this may include inappropriate sampling times and test sensitivity.

CHAPTER 1
INTRODUCTION

Overall calving rates in beef cattle in the Gulf states are estimated at between 70 and 75% (Florida Agriculture Statistics, 1985-1993); a figure well below optimum. Increasing reproductive efficiency in a cost-effective way, would increase efficiency of beef production, profitability, and decrease pressures upon scarce natural resources. Most beef cattle in the US use natural breeding where the bull plays a pivotal reproductive role. In turn, the reproductive efficiency of bulls is related to their spermatogenic capabilities (Chenoweth, 1994a). Elevated environmental temperatures can impair spermatogenesis in bulls which leads to decreased semen quality with associated lowered fertility (Casady et al., 1953; Skinner and Louw, 1966; Rhynes and Ewing, 1973; Meyerhoeffer et al., 1985; Ax et al., 1987). In addition, bull spermatogenesis has been reported to be adversely affected by elevated body temperatures, toxins, and viral disease (Casady et al., 1953; Austin et al., 1961; Johnston et al., 1963; Barták, 1973; Burgess and Chenoweth, 1975; Larsen and Chenoweth, 1990; Vogler et al., 1993; Chenoweth et al., 1994). Common semen characteristics in affected bulls include lower sperm numbers, lower motility, increased abnormal sperm, and increased dead sperm, all of which have been associated with infertility (Saacke, 1982). Breed differences occur in susceptibility to heat stress, with less adverse reproductive effects being reported in males of B. indicus

breeds compared to those of B. taurus derivation (Chenoweth, 1991). In addition to the adverse reproductive effects attributable to specific stressful events, sperm output is generally lower than expected from theoretical extrapolation of spermatogenic precursors in the mouse, rat, and human with such findings being associated with germ cell degeneration and depletion (Oakberg, 1956; Russel and Clermont, 1977; Johnson et al., 1984). In the rat, the cells most implicated in this degenerative process include spermatogonia (Kerr, 1992) and spermatocytes (Brinkworth et al., 1995).

Lowered sperm output is usually associated with degeneration of the spermatogenic epithelium, a process in which apoptosis (or programmed cell death) probably plays an important role. Apoptosis has been observed and studied in a variety of cells and tissues including those of the developing embryo, regressing corpus luteum, lymph nodes, gastrointestinal tract, tumors, and testes (Wyllie et al., 1980; Bowen and Bowen, 1990; Miething, 1992; Palumbo and Yeh, 1994). In structural terms, apoptosis is characterized by condensation of chromatin and cytoplasm, breakage of DNA in a characteristic ladder pattern on electrophoresis, and the formation of apoptotic bodies which are subsequently phagocytosed by macrophages or Sertoli cells in testes. Apoptosis has been observed in the testes of guinea pigs, rats, hamsters, deer, cattle, and other mammals (Allan et al., 1992; Shikone et al., 1994; Higst and Blottner, 1995). Testicular apoptosis in rats has been induced by elevated temperatures or by withdrawal of GnRH, LH, FSH, and testosterone (Tapanainen et al., 1993; Shikone et al., 1994). It has also been associated with an increase of diadem/crater defects in ejaculated sperm, and observed during seasonal testicular regression in the roe deer (Hingst and Blottner,

1995). Increased diadem/crater defects of ejaculated sperm have also been associated with elevated body temperature (Malmgren and Larsson, 1985), scrotal insulation (Vogler et al., 1993) and spermatoxic agents such as ethylene dibromide (Courtens et al., 1980; Eljack and Hrudka, 1979). Such defects are first detectable by microscopy in round spermatids (Larsen and Chenoweth, 1990).

A common mechanism underlying these different disruptions to spermatogenesis may only be hypothesized at present. However, it is well established that an imbalance between the generation of reactive oxygen species (ROS) and their elimination can result in cellular damage (Cagnon et al., 1992). Oxidative stress has been reported to induce apoptosis in thymocytes and embryonic cortical neurons (Ratan et al., 1994; Wolfe et al., 1994). Excessive free radical formation (or lack of elimination) has also been proposed as a major cause of cell damage resulting from heat shock (Loven, 1988). Free radicals can react with lipids to form toxic by-products such as lipid hydroperoxidase, epoxides, and aldehydes that react with other lipids to cause membrane lipid peroxidation and disruption of membrane integrity (Savanian and Hochstein, 1985; Halliwell, 1987; Radi et al., 1991). Free radicals can also react with proteins and purine and pyrimidine bases to cause proteolysis and oxidation (Salo et al., 1990; Fraga et al., 1990). Sperm contains enzymes implicated in the neutralization of ROS, and damage to sperm occurs when the equilibrium is disturbed between the amounts of ROS produced and the scavenging mechanisms, leading to lower sperm motility and viability as well as damage to the axoneme (Cagnon et al., 1992).

Damage to the spermatogenic epithelium of bulls caused by gossypol, a free radical inducer and sperm axoneme disrupting agent, has been effectively countered with antioxidant administration in the form of vitamin E (Velasquez-Pereira et al., 1995).

Recognition of the role of apoptosis in spermatogenic dysfunction introduces new possibilities for the diagnosis and possible prevention of male infertility. However, in order to use this criterion in the evaluation of spermatogenesis, important prerequisites to consider include the establishment of quantifiable links between testicular stress, spermatogenic dysfunction, and apoptosis, and the identification of predictable markers in ejaculated semen and/or sperm. A potential candidate in this latter group include elevated levels of hsp70 in semen.

The prospect of improving reproductive efficiency in beef herds by boosting bull fertility is attractive, particularly if it can be achieved with minimal cost and intervention. Bulls often suffer decreased semen quality and sperm output during the hottest months of the year with deleterious effects upon both natural and artificial breeding programs. A better understanding of the mechanisms involved in spermatogenic dysfunction could allow protective mechanisms and strategies to be devised.

The objective of this work was to determine the effectiveness of testicular stressors (testicular insulation, E. coli endotoxin, and gossypol) to induce spermatogenic apoptosis and to compare this with summer environmental effects on spermatogenesis in Florida. Possible differences between B. taurus and B. indicus genotypes in their susceptibility to environmentally induced spermatogenic apoptosis and efficiency were investigated. To achieve these objectives, methods of identifying and quantifying

spermatogenic apoptosis were compared with quantitative estimates of spermatogenesis. Methods of alleviating or preventing stress effects on bull spermatogenesis, such as the use of antioxidants were investigated. Two general hypotheses were tested in this study; first, that stressors will increase spermatogenic apoptosis in treated bulls, and second, that spermatogenic apoptosis will be lower in tropically adapted than in temperate breeds during periods of heat stress.

CHAPTER 2
REVIEW OF LITERATURE

Causes of Bull Infertility

Infertility in the bull may be caused by a variety of factors including genetic problems, cryptorchidism, elevated temperatures, disease, toxicity, and others (Andersson et al., 1990; Larsen and Chenoweth, 1990; Chenoweth et al., 1994; Marcus et al., 1997; Steffen, 1997).

Genetic or congenital problems reported to cause infertility in the bull have included chromosomal abnormalities, spermatozoal abnormalities, cryptorchidism, inguinal hernia, testicular hypoplasia, segmental aplasia/hypoplasia of the Wolffian duct system, persistent penile frenulum, as well as others (Andersson et al., 1990; Mcfeely et al., 1993; Chenoweth et al., 1994; Marcus et al., 1997; Steffen, 1997). Also, elevated temperatures, both environmental and experimental have been reported to induce infertility in the bull (Casady et al., 1953; Johnston and Branton, 1953; Johnston et al., 1963; Sidibé et al., 1992; Vogler et al., 1993). Diseases such as ephemeral fever, eperythrozoonosis caused by Eperythrozoon wenyonii infection, and Haemophilus somnus infection have been associated with bull infertility (Burgess and Chenoweth, 1975; Barber et al., 1994; Montes et al., 1994). Toxicity has also been associated with infertility by causing spermatozoal abnormalities, decreasing sperm production, or a

combination of both. For example, administration of dexamethasone or ethylene dibromide has been associated with crater defects of sperm (Coulter, 1976; Eljack and Hrudka, 1979; Courtens et al., 1980). Another toxic agent associated with bull infertility is gossypol, a component of cotton seed which has been associated with increased spermatozoal malformations and diminution of spermatogenesis (Chase et al., 1994; Chenoweth et al., 1994; Velasquez-Pereira et al., 1995).

Causes of Spermatogenic Dysfunction

Effect of Stressors on Spermatogenesis

In natural breeding systems the bull plays a pivotal role in beef production. Suboptimal bull fertility undoubtedly contributes significantly to the relatively low calf crop reported for Gulf States such as Florida (Florida Agricultural statistics, 1985-1993). A number of stressors can adversely affect bull fertility, including elevated temperatures and disease. High ambient temperatures can compromise spermatogenesis in the bull. These adverse effects are often associated with semen changes including lowered sperm concentration, motility and membrane integrity, as well as increased morphological abnormalities. All of these effects have been associated with lowered fertility (Casady et al., 1953; Johnston et al., 1963; Ax et al., 1987; Wolfe et al., 1993). The spermatogenic ephitelium responds to stressors in a stereotyped manner, with disruption of chromatin condensation being a common manifestation. This in turn can lead to the diadem/crater defect in ejaculated sperm, for which earliest evidence may be found in round spermatids (Larsen and Chenoweth, 1990). This defect is considered to represent a morphological

marker for a wide variety of testicular insults, having been induced by scrotal insulation (Vogler et al., 1993), ethylene dibromide toxicity (Courtens et al., 1980), seasonal changes (Haigh et al., 1984), and administration of dexamethasone (Coulter, 1976). Another important morphological marker commonly associated with spermatogenic damage is disruption of the sperm midpiece, varying from subtle gaps and discontinuities to more bizarre manifestations. Such midpiece abnormalities may be observed following gossypol-induced spermatogenic damage in bulls (Chenoweth et al., 1994).

Effect of Elevated Temperatures on Spermatogenesis

Disruption or diminution of spermatogenesis in bulls has been induced either experimentally or naturally by unilateral castration, diseases such as ephemeral fever, or by elevated temperatures secondary to scrotal insulation, environmental chambers, and cryptorchidism (Barták, 1973; Burgess and Chenoweth, 1975; Ross and Entwistle, 1979; Wolfe et al., 1993).

The combination of elevated environmental temperatures and high humidity can also compromise spermatogenesis in bulls (Johnston and Branton, 1953; Casady et al., 1953; Johnston et al., 1963). For example, Johnston and Branton (1953) reported that fertility of dairy bulls declined during the summer months in Louisiana, and spermatogenesis was lowered in Guernsey bulls when bulls were exposed to continuous elevated temperatures using environmental chambers (Casady et al., 1953). Also, Johnston et al. (1963) exposed purebred Holstein Friesian, Brown Swiss and Red Sindhi crossbred bulls to a maximum of 104° F and 54% relative humidity and a minimum of

82° F and 72% relative humidity for an 8 h period every day for 7 days using environmental chambers. Elevated temperature treatment resulted in lower sperm motility, concentration, and total number of spermatozoa. Also, an increase in spermatozoal abnormalities were observed 4 to 6 weeks after heat stress. When initial motility, percentage of abnormal spermatozoa, sperm concentration, and total number of spermatozoa, obtained 3 weeks prior to heat stress treatment were compared with values obtained 4 to 6 weeks after heat stress treatment, motility declined ($P<0.05$) from 100 to 61%, 100 to 81%, 100 to 81%, and 100 to 92% in Holstein, R. Sindhi x Holstein, Br. Swiss and R. Sindhi x Br. Swiss, respectively. Spermatozoal concentration declined from 1194 to 582, 1239 to 947, 935 to 420, and 1126 to 789 million spermatozoa/ml for the same breeds, respectively. Total number of spermatozoa per ejaculate also declined ($P<0.05$) from 6372 to 4927, 13803 to 11949, 4998 to 2817, and 10214 to7791 million for the same respectively, while the percentage of abnormal spermatozoa increased from 9 to 33, 11 to 24, 16 to 28, and 16 to 18 %, respectively.

Meyerhoefer et al. (1985) exposed 16 yearling Angus bulls to either control or heat stress temperatures using environmental chambers. Heat stress bulls were exposed to 35° C for 8 h and 31° C for 16 h during an 8 week period, and control bulls were maintained at 23° C. The heat treatment resulted in lower sperm motility compared with controls ($P <0.01$), and increased percentages of aged acrosomes and abnormal spermatozoa both ($P< 0.05$).

Austin et al. (1961) divided 12 Hereford bulls into control or scrotal insulation (SI) for 48 or 72 h. Scrotal skin temperature was increased by approximately 3° F in

treated bulls compared with control bulls. Also, sperm viability, percent normal, and sperm concentration all decreased (65, 60, and 60 %, respectively) in treated bulls compared to controls. Scrotal insulation for 48 h caused morphological changes in bull spermatozoa (Vogler et al., 1993). In the latter study, abnormalities were first assessed at d 12 after SI and peaked at d 18. The predominant abnormalities at different intervals from SI were tailless sperm that appeared at d 12-15, diadem defects at d 18, pyriform heads and nuclear vacuoles at d 21, knobbed acrosomes at d 27, and "Dag" defects at d 30.

Semen quality in bulls has also been reported to decrease during the hottest months of the year. In Florida, Fields et al. (1979) reported that semen quality and testicular size decreased in Hereford bulls during the summer months. Similar findings were reported by Chase et al. (1993) in Angus but not in Senepol bulls.

Effect of Disease on Spermatogenesis

Disease processes associated with pyrexia can cause impaired spermatogenesis. For example, bovine ephemeral fever, a viral disease of cattle which causes an acute pyrexia, has been associated with increased sperm mid-piece abnormalities (Burgess and Chenoweth, 1975). In another study, Barták (1973), reported that unilateral mumps orchitis resulted in disruption of spermatogenesis in 50% of human patients, as exhibited by increasing oligospermia, necrospermia, and azoospermia. However, it was not readily apparent in either of these studies if the disruption of spermatogenesis occurred as a result of fever, elevation of cytokines, or due to direct toxic or pathogenic effects.

Effect of Toxicity on Spermatogenesis

Compounds such as gossypol, cyclophosphamide, and phthalic acid esters have been reported to disrupt spermatogenesis (Randel et al., 1992; Chase et al., 1994; Chenoweth et al., 1994; Velasquez-Pereira et al., 1995; Richburg and Boekelheide, 1996; Cai et al., 1997).

Gossypol has been shown to have negative effects on male reproduction in humans, monkeys, rabbits, hamsters, rats, mice, and bulls (National Coordinating Group on Male Antifertility Agents, 1978; Chang et al., 1980; Hahn et al., 1981; Saksena and Salmonsen, 1982; Shandilya et al., 1982; Wong et al., 1984; Chase et al., 1994; Chenoweth et al., 1994; Velasquez-Pereira et al., 1995). These effects included lowered sperm motility and sperm counts, increased sperm malformation, and azoospermia (Liu et al., 1987; Risco et al., 1993; Chenoweth et al., 1994). In men, gossypol ingestion at 20 mg/d for 75 days followed by weekly doses of 50 mg, resulted in sperm concentrations lower than 4 million/ml in ejaculates from 99.9% of treated men (National Coordinating Group on Male Antifertility agents, 1978).

When yearling Holstein bulls were fed either 6 or 30 mg total gossypol/kg BW/d for 60 or 42 days, no apparent deleterious effects were observed in either semen quality or spermatogenesis (Jimenez et al., 1989). In contrast, when postpubertal Brahman bulls were fed 8.2 g of free gossypol/day for an 11 week period, the percentage of normal spermatozoa (49 ± 9.8 vs 83 ± 3.2 %; $P=0.001$) as well as sperm motility (52 ± 9.8 vs 82 ± 6.2 %; $P=0.04$) was lower in treated bulls when compared to controls (Risco et al.,

1993). Chase et al. (1994) reported that when Spring-born American Brahman bulls were fed 0, 6 or 60 mg/kg BW/d of gossypol from weaning through puberty, bulls fed 60 mg/kg BW/d reached puberty at an older age than bulls fed 0 or 6 mg/kg BW/d (613 vs 550 or 528 d; P=0.05). However, no differences in semen quality among groups were found. Chenoweth et al. (1994) showed that bulls fed 8.2 g of free gossypol/day during an 11 week period had lower sperm motility, normal spermatozoa, and sperm production (described as daily sperm production and daily sperm production/g of testicular tissue) all ($P<0.05$) than control bulls, and the proportion of sperm midpiece abnormalities was higher in gossypol treated than in control bulls (P=0.05). In a more recent study, dairy bulls fed 14 mg free gossypol/kg BW/d showed a tendency for lower testicular and epididymal weights than controls or bulls fed with the same amount of free gossypol plus 4000 IU Vit E/head/d (245.76 ± 15.58 g vs 262.05 ± 15.58 g and 285.28 g; P=0.1 and 19.44 ± 1.92 g vs 24.68 ± 1.92 g and 25.97 ± 1.92 g; $P \leq 0.1$) (Velasquez-Pereira, 1995).

Other toxic agents such as phthalic acid esters and cyclophosphamide have been associated with decreased fertility by inducing spermatogenic dysfunction that resulted in increased spermatogenic apoptosis in humans and rats. Phthalic acid esters are found in plastizers in food packaging and biomedical devices, and these compounds have been associated with low fertility and testicular atrophy in humans (Thomas and Thomas, 1984; Albro, 1987). Richburg and Boekelheide (1996) treated 28-day-old Fischer rats with mono-(2-ethylhexyl) phthalate (MEHP) at a dose of 2 g/kg per os. Rats killed at 0, 3, 6, or 12 h after treatment showed collapse of Sertoli cell vimentin filaments after 3 hours of MEHP treatment. Sertoli cell function was adversally affected and this resulted

in disruption of spermatogenesis as determined by increased spermatogenic apoptosis.

Cyclophosphamide, a drug used in chemotherapy, has been reported to decrease fertility in humans and animals by inducing oligozoospermia or azoospermia (Qureshi et al., 1972; Trasler et al., 1986; Watson et al., 1985). Cai et al. (1997), treated male Sprague-Dawley rats with cyclophosphamide (70 mg/kg BW). The animals were killed and testes collected at 0, 4, 8, 12, 18, 24, and 48 h after treatment. Apoptosis of germ cells was observed at all stages of spermatogenesis. However, cell death via apoptosis was higher in spermatogonia and spermatocytes in stages I-IV and XI-XIV, suggesting that oligozoospermia and azoospermia resulted from cell death of germ cells.

Effect of Free Radicals on Spermatogenesis

Oxidative stress has been reported to induce apoptosis in thymocytes and embryonic cortical neurons (Ratan et al., 1994; Wolfe et al., 1994). Reactive oxygen species (ROS), which contain an unpaired electron, can increase in tissues as a result of exposure to elevated temperatures (Freeman et al., 1990). These can react with lipids to form toxic by-products such as lipid hydroperoxidase, epoxides, and aldehydes that then react with other lipids to cause lipid peroxidation and disruption of the integrity of membranes (Savanian and Hochstein, 1985; Halliwell, 1987; Radi et al., 1991). Reactive oxygen species can also react with proteins, purine, and pyrimidine bases to cause proteolysis and oxidation (Salo et al., 1990; Fraga et al., 1990). Reactive oxygen species are proposed as major contributors to cell damage resulting from heat shock (Loven, 1988). Laskowska-Lita and Szymanska (1989) used malondiadldehyde (MDA) formation

to measure enzymatic and non-enzymatic lipid peroxidation in bull testis tissue (88.21 ± 3.01 and 103.2 ± 4.23 nmol/mg protein, respectively). However, when 50 μM of the antioxidant glutathione (GSH) was present, non-enzymatic peroxidation MDA concentration dropped from 103.3 ± 12 to 11 ± 0.9 nmol/15 minutes per tissue. The ability of antioxidants to protect spermatogenic tissue against oxidative damage was apparent in a recent study in which the previously established spermatoxic effects of gossypol, presumably caused by stimulating free radical production in bulls (Chenoweth et al., 1994), were countered by administration of vitamin E at 4000 IU/d (Velasquez-Pereira et al., 1995). In this experiment, Velasquez-Pereira (1995), reported that antioxidants (4000 IU Vit E/head/d) added to a diet containing 14 mg free gossypol/ kg/ body weight, provided a tendency of a protective effect evidenced by increased testicle weight (285.28 g in Vitamin E vs 262.05 and 245.67 g, in control and gossypol groups respectively; $P \leq 0.1$).

Effect of Hormonal Changes on Spermatogenesis

Pituitary gonadotropins and testosterone are essential for the normal maintenance of spermatogenesis (Garner and Hafez, 1993). Sertoli cells, which represent the somatic component of the seminiferous epithelium, maintain spermatogenesis under the stimulus of FSH and androgens (Parvinen, 1982; Steinberger, 1991; Garner and Hafez, 1993). Plasma membrane receptors for FSH and intracellular receptors for androgens are present on the Sertoli cell (Means et al., 1976; Heckert and Griswald, 1991; Garner and Hafez, 1993). Androgens are secreted by Leydig cells under the influence of LH (Garner and

Hafez, 1993), and imbalances in either gonadotropins or androgens could disrupt spermatogenesis (Tapanainen et al., 1993; Troiano et al., 1994; Blottner et al., 1996; Brinkworth et al., 1995; Hikim et al., 1997).

Rhynes and Ewing (1973), exposed eight Hereford bulls to 21° C and 50% relative humidity for seven weeks (control period), then four bulls were heat stressed at 35.5° C and 50% relative humidity for seven weeks. Heat stress increased rectal temperature and respiration rate ($P<0.01$) when compared to controls. Also, spermatogenesis as evaluated by semen traits and histology, was impaired by heat treatment, and testosterone plasma concentration declined to 43% of control levels ($P<0.01$) during the first two weeks of heat treatment.

In another study, Sidibé et al. (1992) treated four Swedish Red and White bulls with 96 h of scrotal insulation. Heparinized blood was taken every 2 h for a 24 h period at two week intervals for testosterone, LH, and cortisol determination. Testosterone levels had a tendency to decrease and LH to increase during the period of severe testicular degeneration, whereas the opposite was observed during the period of testicular regeneration. Cortisol levels decreased significantly when bulls were analyzed individually at 10 and 15 weeks after scrotal insulation, suggesting that testicular degeneration is associated with changes in testosterone and LH levels.

Spontaneous Degeneration or Germ Cell Death

Spontaneous death of testicular germ cells appears to be common in many species (Roosen-Runge, 1973; Clermont, 1972). In the absence of overt stress, sperm output is often lower than expected in mice, rats, and humans when compared with the theoretical number that should be obtained from spermatogonial precursors (Oakberg, 1956; Russell and Clermont, 1977; Johnson et al, 1984; Kerr, 1992). The reason for such wastage is not known. The contribution of apoptosis to spermatogenic reduction is unknown and may only be hypothesized at present, even though apoptosis is often observed in spermatogenic tissue. Kerr (1992) described spermatogonial deletion as a cause of testicular germ-cell loss in rats, an effect presumably mediated by apoptosis (Allan et al., 1992). Spontaneous degeneration of spermatogenic cells has also been reported to occur in humans (Johnson et al., 1984) with affected cells displaying characteristics of testicular cells undergoing apoptosis (Miething, 1992; Tapanainen et al., 1993; Brinkworth et al., 1995). Miething (1992) reported that degenerating prespermatogonial germ cells in immature golden hamsters showed increased nuclear and cytoplasm staining intensity characteristic of decondensation. Here the nucleus degenerated into multiple fragments with formation of apoptotic bodies (with or without chromatin) which were eventually phagocytosed by Sertoli cells. In another study, Gorczyca et al. (1993) used human sperm to analyze DNA strand brakes by labeling 3'-OH with dUTP. A correlation of $r=0.87$ was observed between those cells with DNA strand brakes and cells that expressed high sensitivity to DNA denaturation, with the characteristics of this latter group resembling those of apoptotic somatic cells.

Effect of Gossypol on Reproduction

Gossypol is a toxic polyphenolic pigment produced in the pigment glands of roots, leaves, stems and seeds of the cotton plant (Berardi and Goldbleatt, 1969). Gossypol has been related to reproductive problems in several species including humans, rodents and cattle (Liu, 1957; Liu and Segal, 1985, Jimenez et al., 1989; Randel et al., 1992; Chase et al., 1994; Chenoweth et al., 1994). It has been reported that gossypol increases free radical production (de Peyser et al., 1984; Barhoumi and Burghardt, 1996), suggesting that free radicals play an important role in gossypol toxicity. Male effects have been discussed previously.

In female mice, when gossypol acetic acid was administered orally in doses of 40 or 80 mg/kg body weight/d, pregnancy rates decreased from 90% in controls to 60 or 10% in gossypol treated animals respectively (Hahn et al., 1981). In another study, Lagerlof and Tone (1985), reported a decrease in pregnancy rates (89% in controls vs 60% in treated group) in female rats treated orally with gossypol acetic acid (20 mg/kg body weight/d). It has also been reported that gossypol decreased the ability of murine embryos to develop in vitro. In this study (Lin et al., 1989), development scores of embryos were significantly decreased ($P<0.01$) after 72 h of culture from 2.6 in control to 1.8 in embryos treated with 5.3 ng of gossypol compared with controls. Similar results were reported in bovine embryos by Zirkle et al. (1988). Here, embryos were cultured with 0, 1, 5, 10, or 30 μg of gossypol, resulting in lower final development scores in treated than in control embryos (4.1, 3.3, 2.5, 0.4 or 0, respectively; $P<0.05$).

Characteristics of Apoptosis or Programmed Cell Death

Morphologically, apoptosis is characterized as occurring in a relatively small proportion of cells. In general, cells exhibiting apoptosis undergo shrinkage and condensation of chromatin and cytoplasm with chromatin fragmentation (Brinkworth et al., 1995). Eventually, the cell disrupts into randomly distributed organelles, bounded by membranes that are termed "apoptotic bodies." After apoptotic bodies are formed, affected cells are generally phagocytosed by macrophages. With light microscopy, apoptotic liver cells are seen to be surrounded by an abnormal halo and normal tissue, and exhibit condensed basophilic chromatin and eosinophilic cytoplasm. Apoptotic bodies may or may not contain chromatin, and affected cells are phagocytosed by macrophages or epithelial cells (Alison and Sarraf, 1992). Microvilli and junctions of plasma membranes are lost. The chromatin condenses and nuclear crescents are pushed to the periphery of the nucleus resulting in obscure nuclear pores and frequent blebbing of the nuclear membrane. Subsequently, nuclear fragmentation occurs and the nucleolus progressively disintegrates into osmiophilic granules. Integrity of the organelles is maintained, and mitochondria are still active until late stages of apoptosis. Brinkworth et al. (1995) suggested that testicular apoptosis may take a form characterized by cellular swelling and the appearance of decondensed, homogenous chromatin. In apoptosis, DNA is cleaved in an incremental manner of multiples of 180-200 base pairs associated with each nucleosome, giving a characteristic ladder pattern on gel electrophoresis. In apoptotic immature rat thymocytes, disruption of double-stranded DNA leads to the

formation of oligonucleosomes resulting in characteristic chromatin condensation patterns (Wyllie et al., 1980; Cohen and Duke, 1984; Wyllie et al., 1986). However, absence of this DNA ladder pattern has also been reported in cells undergoing apoptosis (Barbieri et al., 1992; Cohen et al., 1992), leading to some disagreement as to its diagnostic value as a marker for apoptosis.

Characteristics of Necrosis

In necrosis, cell death occurs in response to a wide variety of harmful conditions and toxic substances. Necrosis affects groups of continuous cells and inflammatory reaction usually develops in the adjacent viable tissues in response to the related cellular debris. Necrosis is characterized by swelling of the cytoplasm and organelles which leads to organelle dissolution and rupture of plasma membrane. These allow the cellular contents to leak out into the extracellular space, damaging neighboring cells, and the DNA is randomly cleaved by lysosomal deoxyribonuclease resulting in DNA fragments of different sizes (Wyllie, 1981; Allan and Harmon, 1986).

Genes Reported to Play a Role in the Regulation of Apoptosis

Apoptosis is a gene-regulated process. Apoptosis is observed in different cells and organs such as the developing embryo, immune system, ovary, testes, and others. In order to study the genes involved in the regulation of programmed cell death, the nematode C. elegans has been used (Ellis et al., 1991). In C. elegans, several genes have been mapped, and a series of genes have been described at different stages of apoptosis (Hale et al., 1996). In this nematode, Egl-1, ces-1, and ces-2 genes have been described

as genes that specify cell death (Ellis et al., 1991; Hale et al., 1996). In contrast ced-3, ced-4, ced-8, and ced-1 are genes that stimulate programmed cell death or apoptosis (Hale et al., 1996). Ced-9 is the gene that inhibits or suppresses programmed cell death in the C. elegans (Hale et al., 1996). In this nematode, genes involved in the phagocytosis of dead cells or apoptotic bodies are ced-1, ced-6, ced-7, ced-2, ced-5, and ced-10. Nuc-1 is the gene that regulates digestion of dead cells (Hale et al., 1996). The C. elegans genes are important for the study of mammalian apoptosis since there is homology between mammalian genes involved in apoptosis and the genes regulating apoptosis in this nematode (Hale et al., 1996).

In different species, including mammals, different genes have been described as programmed cell death inducers. Tumor suppressor p53, Apo 1 (FAS mediated pathway), nedd2, and ICE are genes that induce apoptosis in mammals (Donehower et al., 1992; Hollstein et al., 1991; Kumar et al., 1992; Kumar et al., 1994; Nagata and Golstein, 1995; Yuan et al., 1993). In the ced-9 family, a large groups of genes have been described that inhibit or suppress programmed cell death,. In humans, Bcl-2, Bax, Bcl-x, Mcl-1, and Bak-2 are included in the ced-9 gene family (Boise et al., 1993; Chittenden et al., 1995; Kozopas et al., 1993; Oltvai et al., 1993; Tsujimoto and Croce, 1986). In murines, ced-9 related genes, homologous to human genes have been described as well as 2 murine specific genes (A1 and Bad) (Lin et al., 1993; Yang et al., 1995). Also, viral genes like Epstein-Barr virus (BHRF1), African swine fever virus (LMW5-HL), and herpes virus Samiri (ORF16) have been described to induce apoptosis (Pearson et al., 1987; Neilan et al., 1993; Smith, 1995).

Detection of Apoptosis in Testicular Tissue

The determination of apoptosis in testicular tissue has been made with use of several methods including histomorphology of chromatin fragments, immuno-histochemical in-situ end-labeling of fragmented DNA, and radioactive DNA-fragmentation (Allan et al., 1992; Tapanainen et al., 1993; Troiano et al., 1994). However, quantification of apoptosis has been difficult with the above methods. In a more recent report (Hingst and Blottner, 1995), testicular apoptosis in sexually active guinea pigs, roe deer, and bulls was quantified in units per mg of testis (U/mg) using an ELISA test (7.08 ± 1.95, 16.32 ± 3.45, and 29 ± 7.1 U/mg testis, respectively). In another study, Blottner et al. (1995), using the same ELISA procedure, reported testicular apoptosis in the brown hare during the testicular proliferation phase of 14.16 ± 2.12 U/mg testis. Another procedure is the in-situ "Tunel" staining method which labels newly formed 3'-OH DNA ends of fragmented DNA in-situ, making possible the identification of specific cell types undergoing apoptosis (Li et al., 1995; Richburg and Boekelheide, 1996; Hikim et al., 1997).

Apoptosis in Testicular Tissue

Although apoptosis may be particularly relevant as a putative cause of spermatogenic dysfunction in the male, relatively little is known concerning the causes or mechanisms of apoptotic processes in the male gonad, or their role in male infertility. Testicular apoptosis may be induced by a variety of agents. In the rat testis, increased temperature as a result of cryptorchidism resulted in increased numbers of apoptotic germ

cells (Shikone et al., 1994). Hormonal events influence apoptosis. Blottner et al. (1995) reported that apoptosis in testes of roe deer was significantly higher during the nonbreeding season than during the breeding season. Interestingly, Haigh et al. (1984) observed an increase in diadem/crater defects of sperm in wapiti, also during the transitional period. Apoptosis in testicular tissue has also been reported after withdrawal of pituitary gonadotropins and testosterone. In the rat, testicular apoptosis increased both after hypophysectomy and following administration of a GnRH antagonist (Tapanainen et al., 1993). However, when hypophysectomized rats were treated with FSH-CTP, hCG, or testosterone, hypophysectomy-induced apoptosis was less evident (16, 49, and 25% respectively of levels in non-treated, hypophysectomized rats) (Tapanainen et al., 1993). Troiano et al., (1994) reported that apoptosis of haploid germ cells in the adult rat increased after testosterone withdrawal. Brinkworth et al. (1995) treated adult male rats with a GnRH antagonist or with methoxyacetic acid, a highly toxic agent for rat pachytene spermatocytes. The GnRH antagonist decreased gonadotropin secretion, as expected, whereas methoxyacetic acid did not cause significant hormonal changes. Both treatments induced the characteristic DNA ladder pattern of apoptosis, as determined by electrophoresis, and increased numbers of degenerating apoptotic germ cells, although some differences occurred in the types of spermatogenic cells affected.

Germ Cell Types Involved in Apoptosis

All germ cells are capable of undergoing apoptosis. However, some types appear to be more susceptible than others, or at least they have been more commonly reported to undergo apoptosis.

In the golden hamster, prespermatogonial cells have been reported to undergo apoptosis between 14 days post conception and 13 days post partum (Miething, 1992). In the normal rat testis, type A_2, A_3, and A_4 spermatogonia have been reported to undergo spontaneous apoptosis (Allan et al., 1992). Shikone et al. (1994), observed that experimentally induced cryptorchidism in 22 d old Sprague-Dawly rats resulted in apoptosis of primary spermatocytes. However, when adult Sprague-Dawly rats were treated with saline, 112.5 μg of GnRH antagonist, or 650 mg of methoxyacetic acid daily for 14 days, A type spermatogonia, as well as leptotene, zygotene, early pachytene, secondary spermatocytes, and spermatids all underwent apoptosis. In GnRH antagonist and methoxyacetic acid treated rats, the types of germ cells undergoing apoptosis were pachytene spermatocytes (all stages), preleptotene stage VII and VIII spermatocytes, and stage XII-XIII spermatocytes (Brinkworth et al., 1995). Richburg and Boekelheide (1996) treated 28 day old Fischer male rats with mono-(2-ethylhexyl) phthalate, resulting in apoptosis of spermatogonia. In a different study, Cai et al. (1997) treated Sprague-Dawley male rats with 70 mg/kg of cyclophospamide, inducing apoptosis in all germ cells. However, apoptosis was most evident in spermatogonia and in stage I-IV and XI-XIV spermatocytes.

Heat Shock Proteins

A variety of cells synthesize heat shock proteins (Hsp) in response to stressors such as hyperthermia (Malayer et al., 1988; Putney et al., 1988; Gutierrez and Guerriero, 1991; Harris et al., 1991; Edwards et al., 1997). Heat shock proteins are important for protein oligomerization, folding, translocation, secretion, and recognition of malfolding proteins, as well as protecting ribosomal RNA (Riabowol et al., 1988; Duncan and Hershey, 1989; Henry and Kola, 1991; Nover and Scharf, 1991).

In the bovine, Hsp70 has been reported to be induced in response to heat shock in endometrial tissue, bovine embryos, lymphocytes, skeletal muscle, brain, kidney, liver, lungs, spleen, testes, and others (Malayer et al., 1988; Putney et al., 1988; Gutierrez and Guerriero, 1995; Edwards et al., 1997). Levels of Hsp70 in bovine tissues are shown in Table 2-1. Also, levels of Hsp70 in lymphocytes cultured at 42° C for 1 h were 5.0 ± 0.36, 4.8 ± 0.36, and 4.2 ± 0.39 μg/mg of protein for Angus, Brahman, and Senepol heifers, respectively (Kamwanja et al., 1994).

Heat Shock Proteins in Testicular Tissue

Spermatozoa possess highly condensed chromatin and are thus unable to undergo transcription. However, O'Brien (1987) identified Hsp and other proteins as being synthesized in mouse leptotene/zygotene spermatocytes, pachytene spermatocytes, and round spermatids. Allen et al., (1988) reported the presence of a 70 Kda protein related to Hsp 70, a novel protein synthesized in association with germ cell differentiation. Heat shock protein 70 is evident in small amounts in unstressed preleptotene and

Table 2-1. Concentration of Hsp70 in bovine tissues as determined by ELISA.

	Hsp70 Concentration (ng/μg) ± SD	
Tissue	Gutierrez and Guerriero, 1991	Gutierrez and Guerriero, 1995
Brain	1.9 ± 0.15	2.1 ± 0.20
Heart	3.5 ± 0.06	4.1 ± 0.30
Kidney	5.5 ± 0.32	3.6 ± 0.30
Liver	4.7 ± 0.20	3.6 ± 0.70
Lung	3.4 ± 0.63	2.9 ± 0.50
Skeletal Muscle	9.1 ± 0.71	8.7 ± 0.40
Spleen	2.6 ± 0.60	2.4 ± 030
Testes	1.8 ± 0.20	2.6 ± 0.20

leptotene-zygotene spermatocytes, but in larger amounts in pachytene spermatocytes (where most synthesis occurs) and in round spermatids. Heat shock protein 72 has been induced in male mouse germ cells by elevated temperatures (Zakeri et al., 1990). Miller et al. (1992) have identified a number of heat shock proteins in human sperm.

Scrotal Insulation Model

The scrotal insulation model has been widely used to study adverse effects upon spermatogenesis in male ruminants, with its effects on aspects of bull spermatogenesis being particularly well characterized. These effects include reduced spermatogenesis, consistent temporal patterns of occurrence of different sperm abnormalities, and adverse effects on sperm freezability. For example, Austin et al. (1961) compared scrotal insulation times of either 24 h (n=4) or 72 h (n=4) for mature bulls. Both treatments caused a decline in both live and normal sperm to 55% of control levels by 2 weeks following treatment. Mean sperm concentration also declined following both treatments to 60% of control levels at 6 weeks. Scrotal insulation for 6-11 weeks in 10 healthy men, ranging from 19-43 years old, resulted in a depression of spermatogenesis to a mean 14.1% of pre-treatment levels at the 6th week of treatment (Robinson and Rock, 1967).

Ross and Entwistle (1979), using five normal bulls, applied scrotal insulation for either 10 or 20 h. Sperm concentration in ejaculates declined in the 10 h group only, decreasing from weeks 7 to 11 post treatment. These workers also described testicular cell degeneration (and types of cells involved) following scrotal insulation by injection of ^{3}H-thymidine into the spermatic artery. Testicular sections were taken for histology after

1.25-2 h or 13.6-14.5 d of ^{3}H-thymidine infusion and scrotal insulation (10 or 20 h). In both treatments, the number of B-type spermatogonia and young spermatocytes declined in the sections taken around d 14 when compared with the sections taken 1-2 h following ^{3}H-thymidine infusion.

The effect of scrotal insulation on cryopreserved bovine sperm has also been documented. Vogler et al. (1991) reported that scrotal insulation for 48 h in six young bulls lowered sperm viability in semen collected from d 12-39 after insulation. Semen collected at d 3-9 that was frozen/thawed and incubated for 3 h at 37° C exhibited lower spermatozoal viability than semen collected prior to treatment.

Bull sperm abnormalities following 48 h of scrotal insulation were described by Vogler et al. (1993). Significant increases in abnormal sperm became apparent 12 d after initiation of treatment (47.5 ± 27.4%), peaking at d 18 (86.3 ± 9.8 %). Lowest sperm motility occurred 15 d after treatment (42 ± 9.8 %). Different types of sperm abnormalities predominated in a sequential manner beginning with tailless sperm at d 12-15, "diadem" sperm defects at d 18, nuclear vacuoles and pyriforme heads at d 21, knobbed acrosomes at d 27, and "Dag" defects at d 30.

Scrotal skin temperature has been reported to increase as a result of scrotal insulation. Austin et al. (1961) reported a skin temperature of 33.3 ° C immediately after initiation of insulation, with mean scrotal skin temperatures as a result of insulation being 33.9°, 35.4°, 36.1°, and 34.7° at 8 a.m., 12 noon, 4 p.m., and 10 p.m. respectively. Ross and Entwistle (1979) reported a maximum temperature in the scrotal pouch of 35.0 ± 0.5° C, temperature during the period of scrotal insulation. Wildeus and Entwistle (1983a)

applied scrotal insulation for 48 h to bulls reaching scrotal temperatures ranging between 32° to 34° C in controls vs 35.5° to 38° in scrotal insulated bulls, with a mean temperature difference of 4.05° C between scrotal insulated and controls. Sidibé et al. (1993) reported a mean testicular skin of 31.6° C before scrotal insulation and mean temperatures of 34.8°, 35.5°, 35.8°, 36°, and 35.8° C inside the scrotal insulator at 2, 24, 48, 72, and 96 h after initiation of scrotal insulation, with a scrotal temperature increase of 3.2° to 4.4° C. Vogler et al. (1993) reported a mean testicular surface temperature of 34.8° C as a result of scrotal insulation, with the temperature of the testicular surface ranging from 33.3° to 36.4° C.

Overall these reports indicate that scrotal insulation caused an increase in scrotal skin temperature in the bull, and that this increase in scrotal skin temperature is enough to disrupt spermatogenesis in the bull.

Effect of *E. coli* Endotoxin Infusion

This endotoxin is a lipopolysaccharide derived from E. coli 055:B5, which has been used in horses to study peritoneal macrophages and to induce mastitis in the bovine (Morris et al., 1992). In the horse, infusion of E. coli endotoxin causes pyrexia and an increase in plasma concentration of TNF, IL-6, 6-keto PGF1α, thromboxane, and other compounds. Escherichia coli endotoxin increased IL-6 when administered to neonatal foals (Robinson et al., 1993), and when 1000 or 30 ng/kg of endotoxin were administered to the horse, IL-6 increased after 3 h of infusion from 171 ± 10.2 U/ml to 10128 ± 4096 and 1555 ± 1326 U/ml, respectively; TNF was detectable in blood and pyrexia occurred

(MacKay and Lester, 1992). In another experiment (Morris et al., 1992), horses infused with 30 ng/kg of endotoxin showed pyrexia, leukopenia after 1 h, leukocytosis after 8 h, and an increase in IL-6 between 1.5 and 5 h after infusion, peaking between 3-4 h. Endotoxin also caused local increases of IL-6, TNF, and inflammatory signs when infused intra-articularly or into the mammary gland (Hawkins et al., 1993; Shuster et al., 1993).

Effect of Elevated Temperatures on Testosterone Levels

Lowered hormonal levels are associated with increased spermatogenic apoptosis in several species such as deer and rat (Tapanainen et al., 1993; Troiano et al., 1994; Brinkworth et al., 1995; Blottner et al., 1996), and this finding is relevant for bulls in tropical or subtropical regions of the world. Here, heat stress may lower levels of pituitary gonadotropins and testicular androgens (Rhynes and Ewing, 1973; Minton et al., 1981; Sidibe et al., 1992), increasing apoptosis levels in germ cells. Peripheral testosterone levels in bulls have shown variable effects as a result of environmental heat stress. This is possibly because the testicles of bulls, in common with a number of domestic species, have the ability to preserve its temperature (thermoregulation) through complex physical and physiological mechanisms. Rhynes and Ewing (1973) reported that plasma testosterone levels of 8 mature (20-26 months old) Hereford bulls decreased to 43% of control values during exposure to elevated temperature (35.5 ± 1° C) for 7 weeks in environmental chambers following a 7 week control period (21.1 ± 1° C). In contrast, Minton et el. (1981), reported that serum testosterone was not reduced in bulls that were

exposed to 34° C in environmental chambers for 15 days. Peripheral testosterone concentrations have decreased after local heating of the testis. For example, Sidibé et al. (1992), using 3 year old bulls, found that serum testosterone levels decreased by 25% following 96 h of scrotal insulation, reaching lowest levels at 6 weeks following treatment. Setchell et al. (1991) reported that exposure of ram testes to 42° C for 45 minutes resulted in lowered peripheral plasma levels of testosterone and lowered testosterone levels in rete testis fluid. However, plasma testosterone levels in blood collected from the spermatic vein did not differ whereas testicular blood flow was lower in heat treated testicles than in controls. Similar results have been observed in rats. Galil and Setchell (1987) reported lower testosterone concentrations in peripheral blood from testis-heated (41.5-43° C) rats following stimulation with hCG 21 d post heating. In contrast, the testosterone concentration in blood collected from testicular veins after hCG stimulation was higher in heat-treated rats than controls. These results suggested that changes in testosterone concentration resulting from elevated temperatures are caused by changes in testicular blood flow, and changes are not necessarily due to decreased Leydig cell capability to synthesize testosterone.

Testosterone concentrations also decline with testicular involution during the non-breeding season in seasonal breeders. Finnish Landrace and Suffolk rams have lower testosterone concentrations in the non-breeding season than in the breeding season (Schanbacher and Lunstra, 1976). Similar results were reported in roe deer by Blottner et al. (1995), with testosterone concentrations being highest during the breeding period.

Effect of Elevated Temperatures on Gonadotropins

Pituitary gonadotropins can also be adversely affected by elevated temperatures. Minton et al. (1981) exposed eight mature bulls to 34° C using environmental chambers to evaluate the effects of elevated ambient temperature on serum LH before and after challenge with GnRH. Bulls were exposed during a 3 week period at 22° C. After this adjustment period bulls were separated into control (22 ± 1° C) or heat stress (34 ± 1° C) groups for a 15 d treatment period. Blood samples were collected on days -2, 6, and 15 of treatment at 30 minute intervals for a 12 h period, after which the bulls were parenterally administered 200 μg GnRH. Blood samples were subsequently taken every 15 minutes for 1 h and then every 30 min for 5 h. Non-stimulated LH levels were reduced on days 6 and 15 in heat-stressed bulls compared to controls. In addition, LH secretory peaks were reduced in heat-stressed bulls compared with controls on days 6 and 15. However, LH levels in response to GnRH challenge were not reduced by heat stress. In contrast, when testes of rams were heated by immersing the scrotum in a water bath (45° C) for 30 or 45 minutes, plasma LH and FSH concentrations did not differ in heat treated rams as compared to controls (Setchell et al., 1991). This differed from a previous study by Galil and Setchell (1987) where exposure of rat testes to 43° C for 30 minutes caused increases in plasma LH and FSH concentrations from d 7 to d 42 following treatment.

Breed Differences in Susceptibility to Elevated Temperatures

Breed differences in susceptibility to elevated temperatures have been reported between Bos taurus and Bos indicus genotypes. For example, Johnston et al. (1963) exposed purebred Holstein Friesian, Brown Swiss, and Red Sindhi crossbred bulls to a maximum of 104° F and 54% relative humidity and a minimum of 82° F and 72% relative humidity for an 8-h period every day for 7 days using environmental chambers. Crossbred bulls (Red Sindhi x Holstein and Red Sindhi x Brown Swiss) had lower ($P<0.01$) rectal temperature (100°± 0.12 and 102.8°± 0.27; 101.5°± 0.12 and 103°± 0.32 F, respectively) when air temperature was 82° or 104° F than purebred bulls (Holstein, 102.6°± 0.28 and 105.2°± 0.35 F; Brown Swiss, 103.6°± 0.30 and 104° ± 0.34 F). Respiration rates were also lower ($P<0.05$) in crossbred (56 ± 3.9 and 60 ± 5.5 counts/min, respectively) than in purebred bulls (80 ± 3.2 and 75 ± 6.4 counts/min, respectively). Results showed that elevated temperatures and high humidity lowered semen quality, and that these deleterious effects were greater in purebreds than in crossbred bulls.

In Florida, Fields et al. (1979), compared semen traits in yearling Hereford, Angus, Santa Gertrudis, and Brahman bulls for semen traits. In this study, Herefords from Montana showed lower semen quality than Brahman, Angus, Hereford from Florida, and Santa Gertrudis. In addition, breeds with better ability for thermoregulation such as Brahman are also more resistant to the effect of heat stress than more thermosensitive breeds such as the Scottish Highland (Seif et al., 1979). Another example of breed

differences in response to elevated temperatures was observed in Australia, where Turner (1982) measured rectal temperatures in two B. taurus (Hereford x Shorthorn) and four B. indicus x B. taurus lines. In this study the average rectal temperature was 39.8°C, being 0.5° C higher in B. taurus than in B. indicus x B. taurus crosses.

Related differences in response to elevated temperatures have also been reported in Bos taurus and Bos indicus bulls in the tropics. For example, Kumi-Diaka et al. (1981), reported higher sperm abnormalities, lower percentage live-sperm, and lower sperm concentration during the hot periods in Bos taurus than in Bos indicus bulls.

The Senepol is a tropically adapted B. taurus breed developed in St. Croix US Virgin Islands during the early 1900s. Senepol originated from crosses between the N'Dama and Red Poll breeds (Hupp, 1981). The Romosinuano is another tropically adapted B. taurus breed. Romosinuano is a criollo breed native to Colombia (Rouse, 1977). Bos taurus tropically adapted breeds have been reported to be more resistant to heat stress than temperate B. taurus such as the Angus (Chase et al., 1993; Hammond et al., 1996). For example, when temperate B. taurus (Angus) bulls were compared with tropically adapted B. taurus (Senepol) during the Florida summer, Senepol bulls had 0.5° C lower ($P<0.003$) rectal temperature than Angus bulls; semen quality tended to decrease in Angus but not in Senepol bulls (Chase et al., 1993). When Senepol bulls were compared with Holstein bulls (temperate B. taurus) in the semi-arid environmental conditions of St. Croix, Virgin Islands, Wildeus and Hammond (1993) reported that Senepol bulls had lower ($P<0.01$) rectal temperatures than Holstein bulls (39.3 vs 40.0° C), and packed cell volume was higher ($P<0.01$) in Senepol than in Holstein bulls (41.1

vs 35.2%, respectively). Also, Senepol bulls showed overall higher spermatozoa concentration with lower percentage of sperm abnormalities than Holstein bulls ($P<0.05$).

In another study illustrating physiological differences between genotypes, Kamwanja et al. (1994), cultured lymphocytes from Angus (temperate Bos taurus), Brahman (tropically adapted Bos indicus), and Senepol (tropically adapted Bos taurus) heifers (n=12 per breed) to 45° C for 3 h. In this experiment cell death was affected by a breed by temperature interaction ($P<.01$), with cell viability being lower for Angus than for Brahman or Senepol, indicating that lymphocytes from tropically adapted breeds (Brahman and Senepol) were more resistant to heat shock than lymphocytes from Angus. In addition, Hammond et al. (1996) evaluated heat tolerance among temperate B. taurus (Angus and Hereford), B. indicus (Brahman), and tropically adapted B. taurus (Senepol and Romosinuano) heifers. Here, rectal temperature was lower ($P<0.001$) in Brahman, Senepol, and Romosinuano than in Angus heifers (39.6°, 39.2°, and 39.5° vs 40.4° C, respectively), and respiration rates were faster ($P<0.05$) in Angus and Hereford than in Brahman, Senepol, and Romosinuano (69 and 64 vs 36, 57, and 55 respirations per min, respectively). These results suggest that spermatogenesis, a temperature dependent process, could be less disrupted in tropically adapted than in temperate breeds under heat stress conditions.

Hammond et al. (1998) investigated heat tolerance in Tuli x Angus, Senepol x Angus, and Brahman x Angus heifers under environmental conditions in central Florida. In trial 1, 38 Brahman, 21 Senepol, 19 Brahman x Angus, 20 Senepol x Angus, and 20 Tuli x Angus heifers were used. Rectal temperature ($\log_{10}$) on the hottest day did not

differ between Brahman and Brahman x Angus (0.39 ± 0.011 and 0.37 ± 0.016, respectively) or between Tuli x Angus and Brahman x Angus (0.35 ± 0.015 and 0.35 ± 0.015, respecively). Rectal temperature (log_{10}) was lower in Senepol x Angus than in Senepol and Tuli x Angus (0.30 ± 0.015 vs 0.35 ± 0.015 and 0.35 ± 0.015 respectively; $P<0.05$), and respiration rate and blood packed-cell volume were lower ($P<0.05$) in Brahman than in Brahman x Angus. In trial 2, 13 Angus, 35 Brahman, 30 Senepol, 23 Brahman x Angus, 17 Senepol x Angus, and 28 Tuli x Angus heifer were used. On the hottest day of trial 2, rectal temperature (log_{10}) and respiration rate were higher ($P<0.05$) in Angus (0.59 ± 0.017 and 74 ± 2.7, respectively) than Brahman (0.47 ± 0.01 and 39 ± 1.6, respectively), Tuli x Angus (0.47 ± 0.011 and 60 ±1.8, respectively), Senepol x Angus (0.43 ± 0.014 and 55 ± 2.4, respectively), and Tuli x Angus (0.50 ± 0.012 and 48 ± 2.0, respectively). Also, respiration rate was higher ($P<0.05$) in Brahman x Angus than in Brahman. This work indicates that heat tolerance in female crosses between tropically adapted Brahman, Senepol, and Tuli with temperate Angus is similar to that of tropically adapted Senepol or Brahman.

As elevated temperatures disrupt spermatogenesis (Johnston and Branton, 1953; Casady et al., 1953; Johnston et al., 1963; Vogler et al., 1993), it is possible to speculate that non-heat-tolerant breeds such as temperate B. taurus (Angus) are more susceptible to suffer more spermatogenic damage as a result of elevated temperatures than heat-tolerant breeds such as B. indicus (Brahman) or tropically adapted B. taurus (Senepol or Romosinuano). Also, if non-heat-tolerant breeds undergo more spermatogenic damage than heat-tolerant breeds, it is possible that spermatogenic apoptosis could be higher in

temperate B. taurus (non-heat-tolerant) than in tropically adapted or heat-tolerant B. indicus, B. taurus, and B. indicus x B. taurus crossbred breeds. No references were, however, found which addressed this specific question.

CHAPTER 3
ATTEMPTS TO INDUCE AND ASSESS SPERMATOGENIC APOPTOSIS IN BULLS

Introduction

Male infertility has been associated with many stressors such as elevated temperatures, toxic agents, and disease processes (Vogler et al., 1993; Richburg and Boekelheide, 1996; Burgess and Chenoweth, 1975). In the bull, environmental heat stress is probably the most ubiquitous. In the southern subtropical US, cattle fertility is significantly lower than in more temperate zones (Chenoweth, 1994), with heat stress being a great contributor to the problem (Badinga et al., 1985; Weller and Ron, 1992). Heat stress is also implicated as a cause of decreased spermatozoal quality in ejaculates of bulls during the summer months (Fields et al., 1979). Spermatogenic damage in several species follows apoptosis, an active process of gene-directed cellular self-destruction (Kerr and Harmon, 1991). Increased spermatogenic apoptosis has been described in several species as a result of elevated temperature, spermatoxicity, hormonal withdrawal and seasonal testicular involution (Shikone et al., 1994; Brinkworth et al., 1995; Blottner et al., 1996; Hikim et al., 1997).

Although spermatogenic apoptosis has been associated with spermatogenic dysfunction in the male, relatively little is known concerning its causes, mechanisms, and role in male infertility. In many species, the spontaneous death of testicular germ cells

appears to be common (Roosen-Runge, 1973; Allan et al., 1987). Normally, sperm output is often lower than expected in mice, rats, and humans when compared with the theoretical number that should be obtained from spermatogonial precursors (Oakberg, 1956; Russel and Clermont, 1977; Johnson et al., 1984; Kerr, 1992). The contribution of apoptosis to sperm reduction is unknown and may only be hypothesized at present, even though apoptosis is often observed in spermatogenic tissue. Kerr (1992) described spermatogonial depletion as a cause of testicular germ-cell loss in rats, an effect presumably mediated by apoptosis (Allan et al., 1992). Spontaneous degeneration of spermatogenic cells has also been reported to occur in humans (Johnson et al., 1984) with affected cells displaying characteristics of testicular cells undergoing apoptosis (Miething, 1992; Tapanainen et al., 1993; Brinkworth et al., 1995). Miething (1992) reported that degenerating prespermatogonial germ cells in immature golden hamsters showed increased nuclear and cytoplasm staining intensity characteristic of decondensation. He reported that the nucleus degenerated into multiple fragments with formation of apoptotic bodies (with or without chromatin) which were eventually phagocytosed by Sertoli cells.

In the present study, two experiments were designed to test the hypothesis that spermatogenic stress, caused by elevation of either body temperature by E. coli endotoxin, or testicular temperature by scrotal insulation, will increase both spermatogenic dysfunction and spermatogenic apoptosis in bulls. Additional objectives were to test breed differences in susceptibility to spermatogenic apoptosis as a result of scrotal insulation, and to determine changes in levels of spermatogenic apoptosis with respect to time of assessment following elevated temperature.

Materials and Methods

Materials

E. coli 055:B5 endotoxin, Merthiolate, butanol, hydrogen peroxide (30%), and methyl green were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, sodium acetate, Permount, xylene, and triton X-100 were purchased from Fisher Scientific (Fair Lawn, NJ). Dulbeco's phosphate-buffered saline (DPBS) was purchased from Life Technologies Inc. (Grand Island, NY). Cell death detection ELISA-kits were purchased from Boehringer Mannheim Co. (Indianapolis, IN), and the in-situ Tunel staining kits (ApopTag peroxidase S7101) were purchased from Oncor (Gaithesburg, MD). Ethanol was purchased from AAPER Alcohol and Chemical Co. (Shelbyville, KY), and acetic acid was purchased from Scientific Products (McGaw Park, IL).

Experiment 1

Eleven mature Angus bulls (averaging 3 yr of age and 700 kg of body weight) of normal reproductive status were acclimatized for 1 wk, at the College of Veterinary Medicine, University of Florida, Gainesville, Florida, where a breeding soundness evaluation (BSE) was performed (d -3). Bulls were distributed into three experimental groups: 1) control, sterile saline infusion (n=3), 2) E. coli 055:B5 endotoxin infusion (100 ng/kg) (n=4), and 3) scrotal insulation (SI) for 48 h (n=4). All treatments started on day 0, when clinical signs (rectal temperature, respiration, and heart rates) were measured at 0, 15, 30, 60, 120, 240, and 360 min after infusion. Insulation of the scrotum was

achieved by placing the scrotum in a sack made from two layers of waterproof nylon taffeta filled with a 1 cm thick batting (Vogler et al., 1993). The jugular vein was used to infuse the endotoxin or saline by venipuncture.

On d 8, semen was collected using a Lane IIIZ Pulsator (Lane Manufacturing, Denver, CO) in conjunction with a 3 ventral-electrode rectal probe. Immediately after semen collection, sperm motility was evaluated, and a smear stained with nigrosin-eosin (NE) (Society for Theriogenology, Hastings, NE) was prepared. A minimum of 200 spermatozoa per slide were assessed for morphology using oil-immersion bright-field microscopy (x 1000), and abnormal sperm were classified based on the region (head, acrosome, midpiece, or tail) where the lesion occurred (Chenoweth et al., 1994). Also, semen was fixed in buffered isotonic formal-saline (FBS) and examined for abnormalities using differential-phase (DIC) microscopy employing a Zeiss Axioscope microscope (x 1000) (Chenoweth et al., 1994).

Animals were sacrificed on d 10 using standard industry procedures at the University of Florida Meats Laboratory (Gainesville, FL) or at Central Packing Inc. (Center Hill, FL). The timing of sample collection (i.e. collection of testicular tissue on 10 d after initiation of treatments) was based on estimation of optimal timing following the work of Ross and Entwistle (1979), who examined testicular sections taken after 1.25-2 h or 13.6-14.5 d of ^{3}H-thymidine infusion and scrotal insulation (10 or 20 h). In both treatments, the number of B-type spermatogonia and young spermatocytes declined in sections taken at 14 d when compared with sections taken 1-2 h following ^{3}H-thymidine infusion. Thus, affected spermatogenic cells should be detected between 1 and 14 d

following treatment, and in this experiment d 10 was chosen. In the present experiment, testes were collected into plastic bags and immediately transported on ice to the laboratory. The spermatic cord was trimmed, and the intact testis and tunic were weighed. The tunic was then removed and the tunic and the testis without tunic were weighed separately. Testicular circumference was measured, and testis length, depth, and width were obtained using calipers. Paired testicular volume was calculated as the sum of the volume of the right and the left testes. Testes were considered as paraboloids, and their volume was calculated using the equation $v = \pi r^2 h$. Where r = (width + depth) / 4 and h = length (Fields et al., 1979; Chase, et al., 1997). Epididymides were removed, weighed, and mid-parenchymal sections were taken for in-situ Tunel staining, for spermatogenesis quantification, and for ELISA. Mid-parenchymal sections were fixed in Bouin's fixative for histological evaluation of spermatogenesis (andrological evaluation or cell association) and apoptosis.

The cell association assessment (andrological evaluation) was done as described by Berndtson et al. (1987). Briefly, Sertoli cells, type A spermatogonia, pre-leptotene, pachytene primary spermatocytes, and stage 8 round spermatids were counted in 20 cross sections of seminiferous tubules at stage VIII. Only Sertoli cells whose nucleus contained a nucleolus were counted. The resulting counts (crude counts) were used to calculate Sertoli : germ cell ratios. Cells with histological characteristics of apoptosis (basophilic and condensed nucleus with smooth edges) were counted in the 20 tubules used for the cell association assessment.

An in-situ Tunel staining method using the ApopTag S7101 in 10% buffered formalin fixed tissue was used to detect apoptosis (Hikim, et al., 1995). This method is based on the specific binding of terminal deoxynucleotidil transferase (TdT) to the newly formed 3'-OH of cleaved DNA. Testicular tissues were fixed in 10% neutral buffered formalin for 24 h, paraffin-embedded and sectioned. Sections were de-paraffinized by two washes in xylene (slide was dipped in and out once per wash), two washes in 100% ethanol, one wash in 90% and 70% ethanol, and DPBS. Endogenous peroxidase was quenched by using 2% hydrogen peroxide in DPBS and then rinsing twice with DPBS. Slides were placed in equilibrium buffer and then in working strength TdT enzyme. The reaction was stopped by adding working-strength stop/wash buffer. Two drops of anti-digoxigenin-peroxidase were applied to slides, and the peroxidase was detected with diaminobenzidine. Negative controls were prepared by adding distilled water instead of TdT enzyme during the preparation of working-strength TdT. Sections were counter-stained with 0.5% methyl green (w:v in 0.1 M sodium acetate, pH 4), and slides were mounted using Permount. The number of apoptotic cells (stained positive) were counted in 10 seminiferous tubules.

Apoptosis was quantified using a cell-death detection ELISA-kit, using aliquots of homogenized testicular parenchyma in DPBS (Hingst and Blottner, 1995). The assay is a quantitative sandwich-enzyme-immunoassay using monoclonal mouse antibodies against DNA and histones. The specificity of this ELISA permits determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Testicular parenchyma (1 g in 2 ml DPBS) was minced, freeze/thawed (- 20° C/18° to 25° C) 3 times, and

homogenized for 1 min using a Polytron (Brinkman Instruments, Westbury, NY), followed by sonication for 1 min at 4° C and centrifugation at 25000 g for 30 min at 4°C. The supernatant was collected and stored in 100 µl aliquots at -20° C. Samples were run in triplicate. The microtiterplate-modules (MM) were coated with 100 µl of coating solution containing the anti-histone antibody, and cultured overnight at 4°C. Incubation buffer (200 µl) was pipetted into each well, wells were covered with plate cover foils, and incubated at room temperature (18° to 25° C) for 30 min to saturate the non-specific binding sites on the wall. After incubation, the incubation buffer was removed, and MM were washed 3 times with 300 µl washing solution. Then 100 µl of each sample solution containing 10 µg of testis equivalent/100 µl were added to the MM, covered, and incubated for 90 min at room temperature. For background determination, 100 µl of incubation buffer were pipetted into3 wells. Then, 100 µl of anti-DNA-peroxidase were added to the MM and incubated for 90 min at room temperature. After incubation, ABTS® (Boehringer) substrate was added to the wells, incubated at room temperature for 10 min, and the absorbance read at 405 nm. The ELISA test was confirmed by running a standard curve using 0, 2.5, 5, 10, 20, 40, and 80 μg of testis equivalent from 3 different bulls.

Spermatogenesis was quantified by counting elongated spermatids to determine daily sperm production (DSP) and DSP/g (DSPG) of testicular parenchyma (Chenoweth et al., 1994). Briefly, 5 g of testicular parenchyma were thawed and finely minced. The sample was homogenized for approximately 1 min in 25 ml of working solution (0.9% NaCl, 0.05% Triton X-100, and 100 ppm Merthiolate diluted 1:4 with distilled water).

Then, 175 ml of working solution were added and mixed for 1 min. The solution was allowed to settle for at least 1 h, and after the settling period, thoroughly mixed using a magnetic stirrer. Elongated spermatids were counted using a hemocytometer (4 fields), and the values were used to determine DSPG and DSP using the formula:

$$DSPG = AX\ (B+Y) / (\text{Time divisor})Y$$

$$DSP = DSPG\ (0.99Z)$$

Where X = hemocytometer count, Y = parenchyma sample weight, Z = testis parenchyma weight, A = hemocytometer constant, B = dilution factor, and time divisor (5.32 = time divisor for Bos taurus) (Amann et el., 1974).

Experiment 2

This experiment was conducted at the Subtropical Agricultural Research Station (28° 37' N latitude, 82° 22' W longitude), Brooksville, Florida. Bos taurus bulls (18 mo of age; Angus, An, n = 8; Senepol, Se, n = 6; Romosinuano, Ro, n = 4) were allotted to two experimental groups; 1) control (An, n = 4; Se, n = 2; Ro, n = 2) and 2) scrotal insulation (SI) for 48 h (An, n = 4; Se, n = 4; Ro, n = 2). Scrotal insulation began at d 0. Bulls were electro-ejaculated on d -6, d -4, and d 1, and semen was evaluated for motility and for morphology (NE and FBS) as described in experiment 1. Semen samples were collected, placed on ice, and transported to the laboratory at the College of Veterinary Medicine (Gainesville, Florida). Samples were centrifuged for 30 min using a microcentrifuged, the supernatant was collected an kept frozen at - 20° C. Later, samples were shipped overnight on dry ice to the Department of Biomedical Sciences (Ontario

Veterinary College, University of Guelph, Ontario, Canada) for quantification of Hsp70 in semen. Heat shock protein 70 was quantify using an ELISA test (unpublished data, Kamarundi and King, 1998). On d 1, rectal temperature and temperature inside the scrotal insulator was measured using a digital thermometer (HH 21, Omega, Stamford, CT). At d 2, after 48 h of treatment, SI was removed from all animals, and 9 bulls were sacrificed within 2 h (control; An, n = 2; Se, n = 1; Ro, n = 1; and SI; An, n = 2; Se, n = 2; Ro, n = 1). The remaining 9 bulls were sacrificed on d 4 (control; An, n = 2; Se, n = 1; Ro, n = 1; and SI; An, n = 2; Se, n = 2; Ro, n = 1). In a recent study, mono-(2-ethylhexyl) phthalate was shown to induce testicular apoptosis in the rat, with apoptosis increasing after 6 h of treatment (Richburg and Boekelheide, 1996). This suggested that detection of testicular apoptosis should be attempted earlier than in experiment 1. As a result, detection of testicular apoptosis in experiment 2 was attempted either in tissue harvested within 2 h of removal of scrotal insulators (2 d) or within 2 days following this (4 d). After sacrifice, tissues were collected and processed as described in experiment 1.

Statistical Analysis

Data were analyzed by least squares analysis of variance (ANOVA), using the GLM procedure of SAS (SAS, 1989, 1996). In experiment 1, the model included treatment and the error term was the residual. For clinical data, sperm motility, and FBS sperm morphology, treatment, bull within treatment, time, and the interaction of treatment by time were included in the model. The difference in sperm motility was calculated by deducting sperm motility at d 8 from sperm motility at d -3 (d -3 - d 8), and

the resulting values were analyzed. Bull within treatment was used as the error term for treatment, and the residual was used as the error term for the rest of model. In experiment 2, the model included treatment, breed, treatment by breed, day, treatment by day, breed by day, treatment by breed by day, and the residual was used as the error term. Since no significant effects were observed, additional analysis including either treatment, day, and day by treatment, or treatment, breed, breed by treatment were conducted. The difference in sperm motility was calculated by deducting sperm motility at d 1 from the average sperm motility between d -6 and d -4, and the resulting values were analyzed. Data for spermatogenesis was also analyzed for breed, and in this analysis treatment was not included in the model. Data for rectal and scrotal temperatures were also analyzed following logarithmic transformations. All data are presented as least squares means ± SEM (LSMeans ± SEM).

Results

Experiment 1

The effect of E. coli endotoxin infusion (100 ng/kg) on (LSMeans ± SEM) respiration and heart rates as well as on rectal temperature is shown in Figure 3-1. There was an effect of time of assessment on respiration rate and rectal temperature ($P<0.05$ respectively), but not of treatment. Percent motility and FBS morphology values (LSMeans ± SEM) measured three days before the beginning of treatments (d -3) and eight days after beginning of treatment (d 8) are shown in Figure 3-2, Figure 3-3, and Table 3-1, respectively. Sperm motility and FBS sperm morphology (percentage of

normal spermatozoa, primary abnormalities, and secondary abnormalities) did not differ as a result of E. coli endotoxin infusion or scrotal insulation (48 h) treatment or time when compared with control bulls. Least squares means (± SEM) for semen and testicular traits at d 8 in control, E. coli endotoxin infused, or scrotal insulated (48 h) bulls are presented in Table 3-2. There was no effect of treatment on testicular and semen traits. Sertoli cells, germ cells, and ratio (LSMeans ± SEM) are shown in Table 3-3 and Figure 3-4. Sertoli cells, germ cells, and Sertoli:germ cell ratio did not differ among groups (control, endotoxin, or SI). Spermatogenic apoptotic cells (LSMeans ± SEM) determined by histology in control, endotoxin, or SI treated bulls are shown in Table 3-3 and Figure 3-5. Spermatogenic apoptotic cells per seminiferous tubule determined by histology were not influenced by treatment (control, endotoxin, or SI). Results on apoptotic cells per seminiferous tubule (LSMeans ± SEM) determined by tunel stain are shown in Figure 3-6. Number of apoptotic cells (stained positive by tunel) per seminiferous tubule did not differ as a result of treatment.

Confirmation of the ELISA test was done by a standard curve. A standard curve is shown in Figure 3-7. Results showed an effect of concentration ($P< 0.05$) on light absorbance at 405 nm. The ELISA test was able to detect differences between concentrations used (contrast 0 vs 2.5, 5, 10, 20, 40, and 80 μg, $P< 0.05$; 2.5 vs 5, 10, 20, 40, and 80 μg, $P< 0.05$; 5 vs 10, 20, 40, and 80 μg, $P< 0.05$; 10 vs 20, 40, and 80 μg, $P< 0.05$). Least squares means (± SEM) for spermatogenic traits (DSP, DSPG) and spermatogenic apoptosis assessed by cell death detection ELISA in control, E. coli endotoxin infused, or scrotal insulated (48 h) bulls are shown in Figure 3-8.

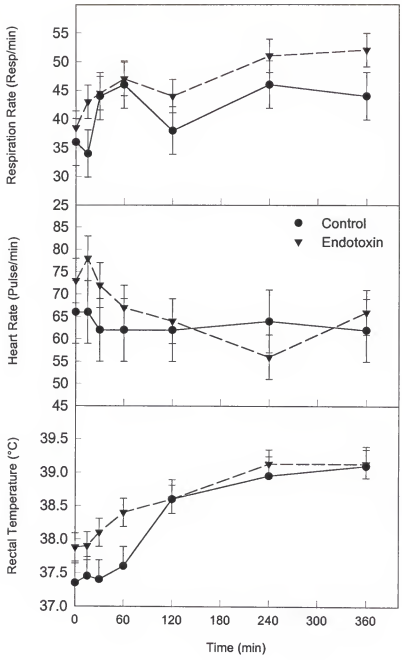

Figure 3-1. Least squares means (± SEM) of the effect of E.coli endotoxin infusion (100 ng/kg) on respiration rate, heart rate, and rectal temperature in bulls. Respiration rate and rectal temperatures were affected by time ($P \leq 0.05$ respectively).

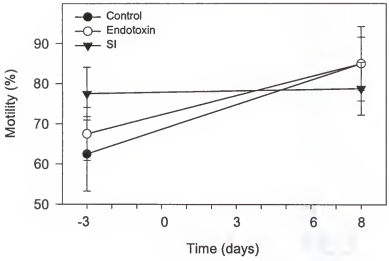

Figure 3-2. Spermatozoal motility (Least squares means ± SEM) in control, E.coli endotoxin infused (100 ng/kg) (endotoxin), or scrotal insulated for 48 h (SI) bulls.

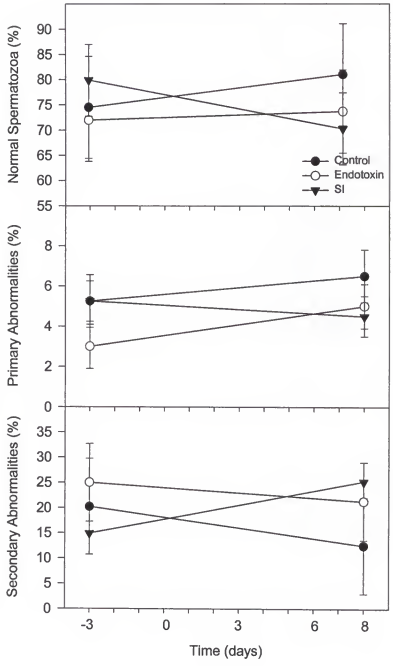

Figure 3-3. Percentage of normal spermatozoa, primary abnormalities, and secondary abnormalities (Least squares means ± SEM) in control, E.coli endotoxin infused (100 ng/kg) (endotoxin), or scrotal insulated for 48 h (SI) bulls.

Table 3-1. Effect of treatment (control, endotoxin, or scrotal insulation) on sperm motility, normal spermatozoa, primary abnormalities, and secondary abnormalities[a].

Item	Control	Endotoxin	Scrotal insulation
Motility			
Day -3	62.5 ± 9.27	67.5 ± 6.55	77.5 ± 6.55
Day 8	85.0 ± 9.27	85.0 ± 6.55	78.8 ± 6.55
Difference (d -3 - d 8)	-22.5 ± 9.86	-2.5 ± 6.97	8.8 ± 6.97
Normal spermatozoa[b]			
Day -3	74.5 ± 10.06	2.0 ± 8.21	79.9 ± 7.11
Day 8	81.0 ± 10.06	73.7 ± 8.21	70.3 ± 7.11
Primary abnormalities[b]			
Day -3	5.3 ± 1.31	3.0 ± 1.07	5.3 ± 0.93
Day 8	6.5 ± 1.31	5.0 ± 1.07	4.5 ± 0.93
Secondary abnormalities[b]			
Day -3	20.3 ± 9.49	25.0 ± 7.75	14.9 ± 6.71
Day 8	12.5 ± 9.49	21.3 ± 7.75	25.3 ± 6.71

[a]Least squares means ± SEM.
[b]Semen fixed with buffered isotonic formal-saline (FBS).

Table 3-2. Semen and testicular traits at 8 d in control, E. coli endotoxin or scrotal insulated (SI) treated bulls[a,b].

	Treatment		
Item	Control	Endotoxin	SI
Normal spermatozoa[c], %	78.1 ± 7.25	72.4 ± 6.28	78.2 ± 6.28
Primary abnormalities[c], %	15.5 ± 6.30	14.2 ± 5.46	15.7 ± 5.46
Secondary abnormalities[c], %	6.4 ± 6.52	13.7 ± 5.65	5.8 ± 5.65
Scrotal circumference, cm	37.0 ± 0.88	35.9 ± 0.62	36.4 ± 0.62
Testicular circumference, cm	20.9 ± 0.38	20.1 ± 0.29	20.1 ± 0.38
Paired testicular vol., cm^3	1298.5 ± 175.05	1124.4 ± 135.60	1090.5 ± 175.05
Paired testicular wt, g	692.8 ± 54.02	635.1 ± 41.84	618.8 ± 54.02
Paired epididymal wt, g	71.0 ± 5.49	75.8 ± 4.25	63.2 ± 5.49

[a]Least squares means ± SEM.
[b]There was no effect on semen and testicular traits.
[c]Semen stained with negrosin and eosin.

Table 3-3. Sertoli cells, germ cells (spermatogonia A, preleptotene, pachytene, and round spermatid stage 8, and Sertoli:germ cells ratio in 20 seminiferous tubules stage VIII in control, E. coli endotoxin infusion (100 ng/kg) (endotoxin), or scrotal insulated for 48 h (SI) bulls[a].

Item	Control	Endotoxin	Scrotal insulation
Sertoli cells	7.1 ± 1.80	8.4 ± 1.56	8.4 ± 1.56
Germ cells	160.9 ± 13.20	169.0 ± 11.43	175.6 ± 11.43
Sertoli:germ cell	22.7 ± 3.78	23.0 ± 3.27	22.5 ± 3.27
Apoptotic cell	0.317 ± 0.0969	0.413 ± 0.0839	0.338 ± 0.0839

[a]Least squares means ± SEM.

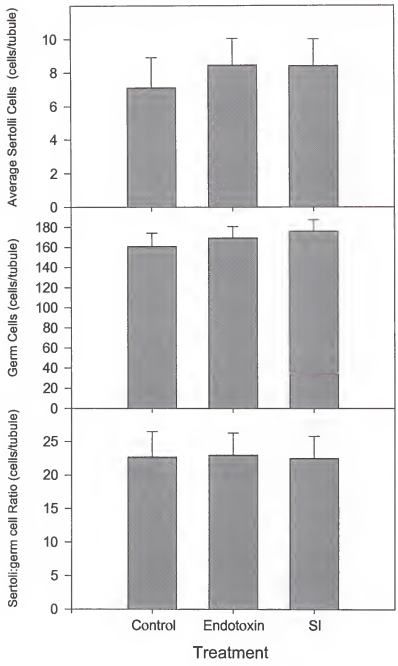

Figure 3-4. Sertoli cells, germ cells (spermatogonia A, preleptotene, pachytene, and round spermatid 8), and Sertoli:germ cells ratio in 20 stage VIII seminiferous tubules (Least squares means ± SEM) in control, E. coli endotoxin infusion (100 ng/kg) (endotoxin), or scrotal insulated for 48 h (SI) bulls.

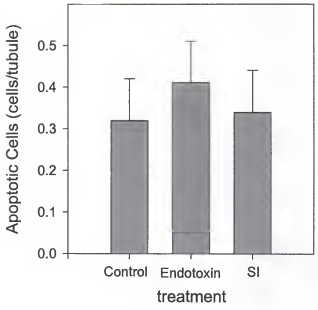

Figure 3-5. Apoptotic cells per stage VIII seminiferous tubule (Least squares means ± SEM) in control, E. coli endotoxin infusion (100 ng/kg) (endotoxin), or scrotal insulated for 48 h (SI) bulls.

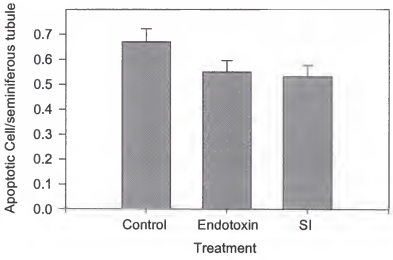

Figure 3-6. Apoptotic cells per seminiferous tubule (LSMeans ± SEM) in control, E. coli endotoxin, and 48 h of scrotal insulation (SI). Spermatogenic apoptosis, assessed by Tunel staining, did not differ among treatments.

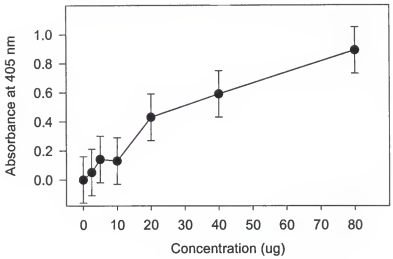

Figure 3-7. Effect of testicular concentration (n=3 bulls) on light absorbance at 405 nm, using a cell detection ELISA. There was an effect of concentration on light absorbance ($P< 0.05$), and orthogonal contrasts 2.5 vs 5, 10, 20, 40, and 80 μg, 5 vs 10, 20, 40, and 80 μg, and 10 vs 20, 40, and 80 μg were significant ($P \leq 0.05$).

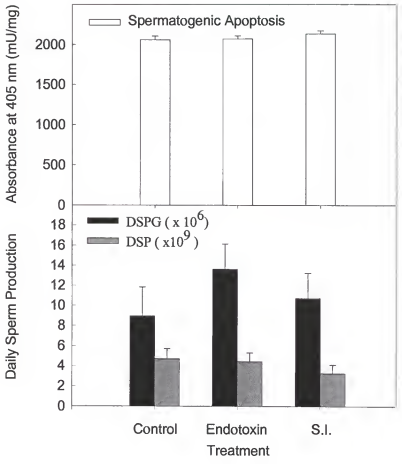

Figure 3-8. Least squares means (± SEM) on the effect of E.coli endotoxin infusion (100 ng/kg) or 48 h of scrotal insulation (SI) on spermatogenic apoptosis, DSPG (daily sperm production/g) and DSP (daily sperm production). There was no effect of treatment on any of the parameters evaluated.

Spermatogenic traits, determined by DSP and DSPG, and spermatogenic apoptosis, were not different among groups. The in-situ 3' end-labeling of apoptotic cells with ApopTag showed staining of less than 1 cell per seminiferous tubule in either control or treated bulls, and no stained cells were observed in negative controls.

Experiment 2

Percent sperm motility (LSMeans ± SEM) on d -6, d -4, and d 1 (in relation to either d 0 or initiation of treatment) is shown on Table 3-4 and Figure 3-9. Sperm motility did not differ as a result of treatment (control or 48 h of SI), breed, or time. Least squares means (± SEM) for FBS sperm morphology (percentage of normal spermatozoa, percentage of primary abnormalities, and percentage of secondary abnormalities) are shown on Table 3-4 and Figure 3-10 and Figure 3-11. The percentage of normal spermatozoa was not affected by treatment or time. However, percentage of normal spermatozoa was affected by breed ($P<0.01$). Angus bulls had less normal spermatozoa when compared with Romosinuano and Senepol ($P<0.01$). Primary abnormalities were not affected by treatment or breed. However, primary abnormalities were influenced by time ($P<0.05$). Primary abnormalities, regardless of treatment or breed, were higher at d 1 ($P<0.01$) when compared with primary abnormalities at d -6 or d -4. Secondary abnormalities were not influenced by treatment or time. However, there was an effect of breed on secondary abnormalities ($P<0.001$). Secondary abnormalities were higher ($P<0.01$) in Angus bulls than in Romosinuano and Senepol bulls.

Table 3-4. Sperm motility, normal spermatozoa, primary abnormalities, and secondary abnormalities in Angus, Romosinuano, and Senepol bulls[a].

Item	Angus	Romosinuano	Senepol
Motility			
Day -6	77.5 ± 7.04	77.4 ± 8.30	73.8 ± 9.96
Day -4	68.8 ± 7.04	79.0 ± 8.30	68.8 ± 9.96
Day 1	75.6 ± 7.04	79.9 ± 8.30	81.3 ± 9.96
Difference[b]	-2.6 ± 4.43	-1.9 ± 5.42	-10.1 ± 6.26
Normal spermatozoa[c]			
Day -6	67.9 ± 3.06	84.9 ± 4.33	86.3 ± 6.61
Day -4	66.1 ± 3.06	86.8 ± 4.33	84.3 ± 6.61
Day 1	72.1 ± 3.06	85.6 ± 4.33	79.1 ± 6.61
Primary abnormalities[d]			
Day -6	10.1 ± 1.30	4.5 ± 1.84	4.4 ± 1.54
Day -4	9.5 ± 1.30	3.6 ± 1.84	3.8 ± 1.54
Day 1	11.2 ± 1.30	6.0 ± 1.84	10.4 ± 1.54
Secondary abnormalities[c]			
Day -6	21.9 ± 2.90	10.6 ± 4.10	9.3 ± 3.42
Day -4	24.4 ± 2.90	9.6 ± 4.10	11.9 ± 3.42
Day 1	16.7 ± 2.90	8.4 ± 4.10	10.5 ± 3.42

[a]Least squares means ± SEM..
[b]Difference on the average of d -6 and d -4 minus d 1.
[c]Orthogonal contrast, breed (Angus vs Romosinuano and Senepol; $P \leq 0.05$).
[d]Orthogonal contrast, time (day 1 vs day -6 and day -4; $P \leq 0.05$).

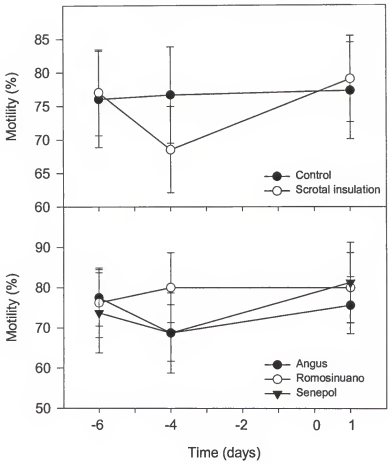

Figure 3-9. Spermatozoal motility (Least squares means ± SEM) in control or scrotal insulated for 48 h (SI) Angus, Romosinuano, or Senepol bulls.

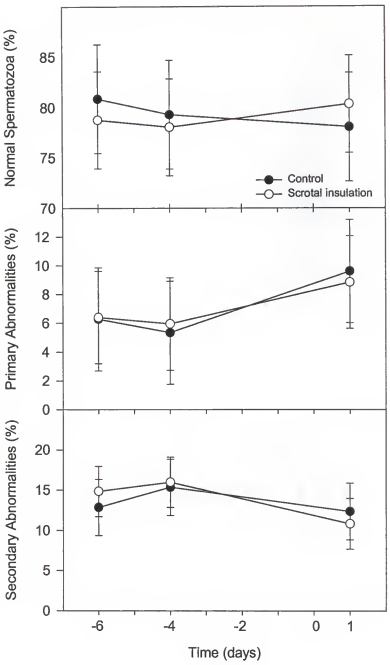

Figure 3-10. Percentage of normal spermatozoa, primary abnormalities, and secondary abnormalities (Least squares means ± SEM) in control or scrotal insulated for 48 h (SI) bulls.

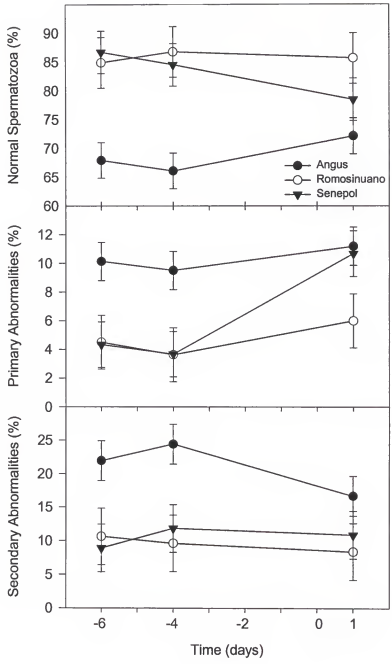

Figure 3-11. Percentage of normal spermatozoa, primary abnormalities, and secondary abnormalities (Least squares means ± SEM) in Angus, Romosinuano, or Senepol bulls.

Table 3-5. Body weight, rectal, and scrotal temperatures in controls and treated bulls of 3 breeds at 24 h following initiation of scrotal insulation (SI) in bulls[a].

	Breed		
Item	Angus	Romosinuano	Senepol
No. of bulls	8	4	6
Body weight (kg)	419.3 ± 12.34[b]	534.8 ± 15.11[c]	522.0 ± 17.45[c]
Rectal Temp (°C)			
Control	39.2 ± 0.26	38.1 ± 0.36	37.5 ± 0.36
Scrotal insulated	38.6 ± 0.26	38.3 ± 0.26	38.0 ± 0.36
Control[b] (Log_{10})	1.6 ± 0.003	1.6 ± 0.005	1.6 ± 0.005
Scrotal insulated[b] (Log_{10})	1.6 ± 0.003	1.6 ± 0.003	1.6 ± 0.005
Scrotal Temp (°C)			
Control	31.2 ± 0.55	30.8 ± 0.78	31.0 ± 0.78
Scrotal insulated	34.4 ± 0.64	33.7 ± 0.55	32.7 ± 0.78
Control[c] (Log_{10})	1.49 ± 0.01	1.5 ± 0.01	1.5 ± 0.01
Scrotal insulated[c] (Log_{10})	1.53 ± 0.01	1.52 ± 0.01	1.5 ± 0.01

[a]Least squares means ± SEM.
[b]Orthogonal contrast, Angus vs Romosinuano and Senepol ($P \leq 0.05$).
[c]Orthogonal contrast, Control vs scrotal insulated ($P \leq 0.05$).

Table 3-5 shows LSMeans (± SEM) for body weight (BW), rectal and scrotal temperatures 24 h after initiation of treatment. Body weight of Angus bulls was lower than Senepol and Romosinuano ($P< 0.01$), and rectal temperature was higher in Angus when compared to Senepol and Romosinuano bulls ($P<0.01$). However, rectal temperature was not affected by scrotal insulation. Scrotal temperature was not affected by breed, and scrotal temperature was higher in scrotal insulated than in control bulls ($P<0.05$).

Least squares means (± SEM) for the effect of treatment (control or 48 h of SI) and day of tissue harvest on semen and testicular traits are shown in Table 3-6. Percentage of secondary abnormalities were higher in both control and SI bulls at d 2 than at d 4 ($P\leq0.05$). In contrast, paired epididymal weight was lower in control than in SI bulls at d 2 and at d 4 ($P\leq0.05$). Percent of normal spermatozoa, percent of primary abnormalities, scrotal and testicular circumference, paired testicular volume, and paired testicular weight were not affected by treatment or day. Least squares means (± SEM) for the effect of breed (regardless of treatment) on semen and testicular traits are shown in Table 3-7. Semen characteristics and paired epididymal weight were not influenced by breed. Scrotal circumference, paired testicular volume, testicular circumference, and paired testicular weight were smaller in Angus bulls than in Senepol and Romosinuano ($P<0.001$ respectively).

Results of the effect of scrotal insulation (SI) on Hsp70 levels (least squares means ± SEM) in bull semen are shown in Table 3-8 and Figures 3-12, 3-13, and 3-14. Heat shock protein 70 levels (μg/ml) were not influenced by breed or treatment ($P>0.05$).

Table 3-6. Semen and testicular traits in control or 48 h scrotal insulated (SI) bulls[a].

	Day 2[b]		Day 4[b]	
Item	Control	SI	Control	SI
Normal spermatozoa[c], %	80.9 ± 7.75	73.2 ± 6.93	72.4 ± 7.75	86.6 ± 6.93
Primary abnormalities[c], %	8.5 ± 5.73	10.5 ± 5.13	21.6 ± 5.73	8.0 ± 5.13
Secondary abnormalities[c,d], %	11.1 ± 2.19	8.2 ± 1.96	6.0 ± 2.19	4.4 ± 1.96
Scrotal circumference, cm	32.9 ± 1.87	33.7 ± 1.67	32.1 ± 1.87	34.1 ± 1.67
Testicular circumference, cm	17.1 ± 0.99	18.2 ± 0.88	16.9 ± 0.99	18.7 ± 0.88
Paired testicular vol., cm^3	723.0 ± 101.16	701.2 ± 90.48	544.7 ± 101.16	826.4 ± 90.48
Paired testicular wt, g	426.8 ± 74.56	486.4 ± 66.69	398.4 ± 74.56	518.1 ± 66.69
Paired epididymal wt, g	48.4 ± 7.84[b]	62.4 ± 7.02[c]	42.5 ± 7.84[b]	60.6 ± 7.02[c]

[a]Least squares means ± SEM.
[b]In relation to day 0 or at the beginning of treatment
[c]Semen stained with nigrosin and eosin.
[d]Contrast, time (day 2 vs day 4; $P \leq 0.05$).

Table 3-7. Semen and testicular traits in control and treated Angus, Romosinuano, and Senepol bulls at 24 h following initiation of scrotal insulation (SI)[a].

	Breed		
Item	Angus	Romosinuano	Senepol
No. of bulls	8	4	6
Normal spermatozoa[b], %	72.1 ± 5.40	88.2 ± 6.61	76.4 ± 7.63
Primary abnormalities[b], %	17.5 ± 4.19	7.5 ± 5.13	7.3 ± 5.93
Secondary abnormalitiesb[b], %	9.8 ± 1.53	4.8 ± 5.93	6.2 ± 2.16
Scrotal circumference[c], cm	30.0 ± 0.72	35.9 ± 0.88	35.7 ± 1.01
Testicular circumference[c], cm	16.2 ± 0.47	19.1 ± 0.58	18.8 ± 0.67
Paired testicular vol.[c], cm^3	533.7 ± 51.10	858.0 ± 62.59	783.1 ± 72.27
Paired testicular wt[c], g	334.9 ± 31.22	573.7 ± 38.23	536.5 ± 44.14
Paired epididymal wt, g	46.1 ± 4.81	60.6 ± 5.89	55.5 ± 6.81

[a]Least squares means ± SEM.
[b]Semen stained with nigrosin and eosin.
[c]Orthogonal contrast, breed (Angus vs Romosinuano and Senepol; $P \leq 0.05$).

Table 3-8. Heat shock protein 70 (μg/ml) in semen of Angus, Romosinuano, and Senepol bulls[a].

Item	Day -6	Day -4	Day 1
Breed			
Angus	260.7 ± 68.48	163.6 ± 68.48	137.9 ± 75.46
Romosinuano	172.8 ± 80.71	173.0 ± 80.71	66.2 ± 80.71
Senepol	309.0 ± 96.85	181.5 ± 96.85	25.8 ± 96.85
Treatment			
Control	288.1 ± 68.00	180.0 ± 68.00	43.4 ± 73.14
SI 48 h	201.9 ± 60.65	159.9 ± 60.65	110.6 ± 60.65
Time [b]	247.5 ± 47.82	172.7 ± 47.82	76.7 ± 49.00

[a]Least squares means ± SEM.
[b]Orthogonal contrast, time (day 1 vs day -6 and -4; $P \leq 0.05$).

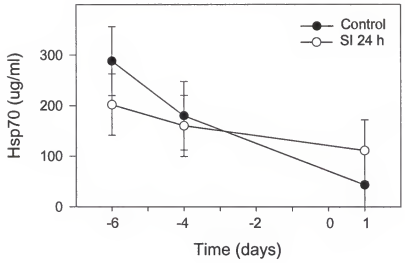

Figure 3-12. Heat shock protein 70 (Hsp70, μg/ml) in semen (least squares means ± SEM) over time in control or scrotal insulation (SI 24 h). Days in relation to initiation of SI treatment. Heat shock protein 70 (Hsp70) in semen was influenced ($P \leq 0.05$) by time but not by treatment.

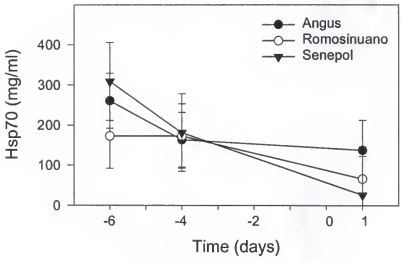

Figure 3-13. Heat shock protein 70 (Hsp70, μg/ml) in semen (least squares means ± SEM) over time (days in relation to initiation of SI treatment), regardless of treatment, in Angus, Romosinuano, and Senepol bulls. Heat shock protein 70 (Hsp70) in semen was influenced ($P \leq 0.05$) by time but not by breed.

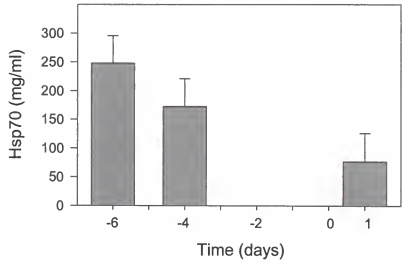

Figure 3-14. Heat shock protein 70 (Hsp70, μg/ml) in semen (least squares means ± SEM) over time (days in relation to initiation of scrotal insulation treatment), regardless of breed and treatment. Heat shock protein 70 (Hsp70) in semen was influenced ($P \leq 0.05$) by time only.

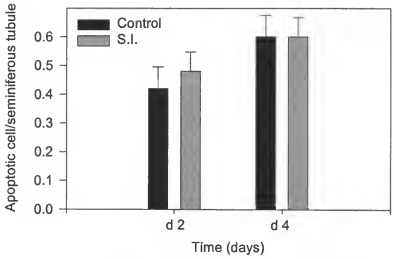

Figure 3-15. Effect of 48 h of scrotal insulation (SI) on apoptotic cells per seminiferous tubule (LSMeans ± SEM) in tissue harvested immediately (d 2) following removal of scrotal insulation, or 2 days later (d 4). Spermatogenic apoptosis, assessed by Tunel staining, was not effected by treatment. However, there was a tendency for an effect of time (P= 0.06).

However, Hsp70 levels were affected by time ($P<0.05$). Results of the number of apoptotic cells per siminiferous tubule after 48 h SI or control bulls are shown in Figure 3-15. Spermatogenic apoptosis, assessed by Tunel staining, was not effected by treatment.

Spermatogenic traits and spermatogenic apoptosis (LSMeans ± SEM) in control and 48 h SI bulls are presented in Figure 3-16. Scrotal insulation showed no effect on DSP, DSPG, and spermatogenic apoptosis. Least squares means (± SEM) for DSP, DSPG, and spermatogenic apoptosis in Angus, Romosinuano, and Senepol bulls are shown in Figure 3-17. Spermatogenic apoptosis and DSPG did not differ among breeds. However, DSP was lower ($P<0.05$) in Angus than in Senepol and Romosinuano bulls.

Discussion

In experiment 1, rectal temperature, heart and respiration rates were affected by time of assessment, but not by treatment, indicating that E. coli endotoxin caused no observable clinical changes compared to control animals. These results suggested that 100 ng/kg, of E. coli endotoxin administered intravenously in the bull, were not sufficient to induce pyrexia, despite achieving such effects in the horse (MacKay and Lester, 1992; Hawkins et al., 1993). In this study, neither semen nor testicular traits were adversely affected by either treatment (E. coli endotoxin or 48 h of scrotal insulation). These semen findings are not surprising as it has been previously shown that abnormal spermatozoa started to appear 12 d after 48 h of SI (Vogler et al., 1993), whereas in the present study semen was only studied up to 8 d after SI, as the main objective was to evaluate

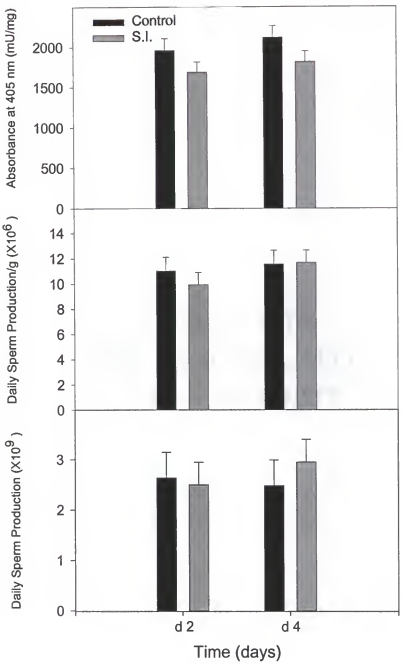

Figure 3-16. Effect of 48 h of scrotal insulation (SI) on spermatogenic apoptosis (absorbance at 405 nm) (LSMeans ± SEM), daily sperm production/g (DSPG), and daily sperm production (DSP) in tissue harvested immediately (d 2) following removal of scrotal insulation, or 2 days later (d 4). There was no effect of treatment on any of the variables.

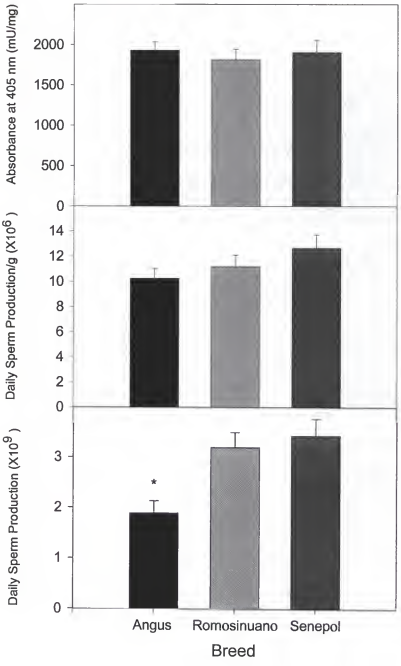

Figure 3-17. Spermatogenic apoptosis (absorbance at 405 nm), daily sperm production/g (DSPG), and daily sperm production (DSP) in Angus, Romosinuano, and Senepol bulls (LSMeans ± SEM). There were no breed effects in spermatogenic apoptosis and DSPG, although DSP was influenced by breed (Angus vs Romosinuano and Senepol; $P \leq 0.05$).

spermatogenic changes, which are generally detectable earlier. In this study, Sertolli:germ cell ratio was higher than the ratios reported (Berndtson et al., 1987; Berndtson and Igboeli, 1989). However, this study is reporting ratios based on crude counts, and ratios reported by Berndtson et al. (1987) and Berndtson and Igboeli (1989) are based on Abercrombie's corrected counts. Testicular characteristics in both experiments were considered to be within the ranges reported for B. taurus bulls in Florida with the exception of Angus bulls used in experiment 2, which had smaller body weights, scrotal and testicular measures than Angus bulls of similar age (Fields et al. 1979; Chenoweth et al, 1996; Chase et al., 1997).

Heat shock protein 70 has been reported to prevent protein denaturation as a result of heat insult. Results in the present study suggest that SI for 24 h did not significantly increase Hsp70 levels in semen, even when SI increased scrotal skin temperature to similar levels previously reported by Vogler et al. (1993). However, Hsp70 was lower at d 1 than at d -6 and d -4, suggesting that some stimulus other than treatment was responsible for decreasing Hsp70. Such a stimulus could be environmental temperatures or animal handling stress. It is possible that the presence of such a stressor increased the demand for Hsp70 resulting in its utilization and subsequent decreased of Hsp70 levels in semen, even when the synthesis of Hsp70 was also increased as result of the stressor. The result of this scenario could be a net decrease of Hsp 70 in ejaculated semen.

Spermatogenic traits (DSPG and DSP) were not affected by any treatment in this study, and values were within previously reported ranges (Weisgold and Almquist, 1979; Amann, 1981; Wildeus and Entwistle, 1982; McCool, 1990; Chenoweth et al., 1994).

However, in experiment 2, DSP for Angus bulls was lower than for Senepol and Romosinuano, although DSPG was not different among breeds. This indicated that spermatogenic efficiency, or production of spermatozoa per unit (g) of testicular parenchyma, was not different among breeds, and that the difference in DSP was most probably due to smaller testicles in Angus bulls compared to Senepol and Romosinuano bull. The lack of effect encountered with quantitative spermatogenic traits was also not unexpected, because it has been reported that elevated temperature treatment (10 or 20 h of SI) decreased the number of type B spermatogonia 14 days after treatment (Ross and Entwistle, 1979). Spermatogenesis in the present study was determined by counting elongated spermatids (DSPG and DSP), and in order to observe a decrease in the number of elongated spermatids as a result of damage to spermatogonia by 48 h of SI, a longer period (more than the d 2, d 4 or d 10 used in the present study) would be necessary.

Spermatogenic apoptosis, as assessed by histology, tunel stain, and cell death detection ELISA, was not influenced by either E. coli endotoxin infusion or 48 h of scrotal insulation in this study. Furthermore, overall levels detected with the ELISA test were lower than those previously reported for cattle, using the same method (Hingst and Blottner, 1995). Possible reasons for these relatively low levels of detectable apoptosis include the following. Firstly, there is the possibility that none of the treatments caused increased testicular apoptosis. This is regarded as unlikely as 48 h scrotal insulation, at least, has consistently produced levels of spermatogenic dysfunction compatible with degeneration and associated apoptosis (Ross and Entwistle, 1979; Vogler et al., 1993). In the present study, scrotal temperatures were significantly higher, after 24 h of scrotal

insulation treatment, in treated than in control bulls. Also, the skin scrotal temperatures achieved in this study are within the ranges reported in the literature (Austin et al., 1961; Ross and Entwistle 1979; Entwistle, 1983a; Sidibé et al., 1993; Vogler et al., 1993). Although relatively few cells stained positive for apoptosis using the ApopTag peroxidase, this was in agreement with low apoptotic levels detected by the cell death detection ELISA. Secondly, it is possible that apoptosis assessment was done before, or after, apoptotic cells had been phagocytosed and rendered undetectable (Richburg and Boekelheide, 1996; Lin et al., 1997). However, in a more recent study, when a GnRH antagonist was used in the rat, testicular apoptosis began to increase after 5 days of treatment, reaching its peak 14 days after treatment (Hikim et al., 1997), suggesting that spermatogenic apoptosis assessment after 48 h of SI, in the bull, should be done following 8 days and before 14 days of treatment. This is because, in the bull, the spermatogonial population has been shown to have declined by d 14 after SI (Ross and Entwistle, 1979).

This series of experiments failed to detect increased testicular apoptosis in bulls after E. coli endotoxin infusion or 48 h of scrotal insulation at 10 d after initiation of treatment or on d 2 or d 4 after initiation of 48 h of SI, suggesting either that these treatments caused no detectable changes in spermatogenic apoptosis, that the detection windows used were inappropriate, or that damage to cells as result of SI or E. coli endotoxin undergo a different type of cell death from apoptosis (Young et al., 1997). Also, the levels of spermatogenic apoptosis observed corresponded to basal levels of apoptosis, resulting probably from spontaneous degeneration of germ cells, that undergo

apoptosis. Since in the rat, it has been shown that germ cells that die during normal spermatogenesis die through apoptosis, suggesting that a basal level of spermatogenic apoptosis is always present during normal spermatogenesis (Blanco-Rodriguez and Martinez-Garcia, 1996).

CHAPTER 4
EFFECT OF DIETS CONTAINING FREE GOSSYPOL AND VITAMIN E ON SPERMATOGENESIS AND SPERMATOGENIC APOPTOSIS IN YOUNG HOLSTEIN BULLS

Introduction

Exposure of bulls to elevated temperatures whether environmental or experimental, results in decreased semen quality (Johnston et al., 1963; Fields et al., 1979; Meyerhoeffer et al., 1985; Chase et al., 1993) and reduced spermatogenesis (Skinner and Louw, 1966). The deleterious effect of elevated temperature on spermatogenesis could be as a result of increased levels of reactive oxygen species in testicular tissue. Reactive oxygen species (ROS), or free radicals, have been proposed as major contributors to cell damage resulting from heat shock by causing lipid peroxidation, protein denaturation, impairment of the cytoskeleton, and disruption of calcium metabolism (Loven, 1988). Gossypol, a toxic polyphenolic pigment produced in the pigment glands of roots, leaves, stems, and seeds of the cotton plant (Berardi and Goldblatt, 1969) has been associated with reproductive problems in several species including humans, rodents, and cattle (Liu, 1957; Liu and Segal, 1985, Jimenez et al., 1989; Randel et al., 1992; Chase et al., 1994; Chenoweth et al., 1994), possibly by increasing free radical production (de Peyser et al., 1984; Barhoumi and Burghardt, 1996). Since gossypol has been reported to increase reactive oxygen species and reduce

antioxidants in hepatocytes and testes in the rat (Bender et al., 1988; Barhoumi and Burghardt, 1996), it is logical to suggest that antioxidants could counter the oxidative damage of gossypol on spermatogenic tissue in bulls. This concept was supported in a recent study where the spermatoxic effects of gossypol in the bull were countered by administration of the antioxidant, vitamin E (Velasquez-Pereira et al., 1995).

In ruminants, feeding diets containing gossypol resulted in decreased spermatogenesis, possibly as a result of damage to germ cells within the germinal epithelium (Randel et al., 1992). In turn, these gossypol damaged germ cells could be removed via apoptosis, resulting in increased levels of spermatogenic apoptosis as a result of gossypol toxicity.

The objective of this study was to evaluate the effect of long term feeding gossypol in cottonseed meal on testicular and spermatogenic traits as well as on spermatogenic apoptosis levels in young Holstein bulls. An additional objective was to determine whether the antioxidant, vitamin E, could counteract the spermatoxic effect of gossypol on testicular and spermatogenic traits and spermatogenic apoptosis which might otherwise be associated with gossypol induced cellular damage.

Materials and Methods

Materials

Reagents were obtained as follows: Merthiolate, butanol, hydrogen peroxide (30%), and methyl green from Sigma Chemical Co. (St. Louis, MO); sodium chloride, sodium acetate, Permount, xylene, and Triton X-100 from Fisher Scientific (Fair Lawn,

NJ); Dulbeco's phosphate-buffered saline (DPBS) from Life Technologies Inc. (Grand Island, NY); cell death detection ELISA-kits from Boehringer Mannheim Co. (Indianapolis, IN); in-situ Tunel staining kits (ApopTag peroxidase S7101) from Oncor (Gaithesburg, MD); ethanol from AAPER Alcohol and Chemical Co. (Shelbyville, KY), and acetic acid from Scientific Products (McGaw Park, IL).

Experimental Design

Young Holstein bulls (n=24; 6 mo of age) were assigned to the following three isocaloric and isonitrogenous dietary groups (each n=8) that satisfied animal requirements for all other nutrients (NRC, 1989); 1) control (CONT), received a supplement containing soybean meal (SBM), corn and 30 IU of vitamin E/kg, 2) gossypol (GOSS), received a supplement containing CSM, corn and 30 IU vitamin E/kg, and 3) gossypol and vitamin E (G+VITE), received a supplement containing CSM, corn and 4,000 IU vitamin E/bull/d. Supplements GOSS and G+VITE were formulated to supply 14 mg of free gossypol/kg body weight (BW)/d (Table 4-1 and 4-2). Animals were housed in 12 pens, 2 animals/pen and 4 pens/treatment, from 6 to 15 months of age. Animals had access to low quality hay in which the vitamin E concentration was less than 9 IU/kg. Supplements were recalculated monthly to ensure that the amount of free gossypol provided on an individual basis was 14 mg/kg BW/d.

Semen was collected 2 weeks prior to sacrifice by electro-ejaculation, using a Lane IIIZ Pulsator (Lane Manufacturing, Denver, CO) in conjunction with a 3 ventral-electrode rectal probe. Immediately after semen collection, a smear stained with

Table 4-1. Initial and final composition of dietary supplements[a]

	CONT		GOSS		G+VITE	
Item	Initial	Final	Initial	Final	Initial	Final
Offered (kg/d)[b]	2.6	5.6	2.7	6	2.7	6
DM (%)	88	88	88	88	88	88
Ingredient[b]						
SBM (%)	59	71	----	----	----	----
CSM (%)	----	----	67	80	67	80
Corn (%)	38	27.5	30	18.5	30	18.5
Limestone (%)	1	0.5	1	0.5	1	0.5
Minerals (%)	2	1	2	1	2	1
Vit. E (IU/kg)	30	30	30	30	1481.5	666.6
Analyses						
(+)-gossypol (%)[c]	0	0	0.33	0.4	0.32	0.4
(-)-gossypol (%)[c]	0	0	0.79	0.98	0.77	0.97
Free gossypol (%)[d]	0	0	0.08	0.11	0.08	0.11
Total gossypol (%)[d]	0	0	1.06	1.16	1.06	1.14

[a]CONT = soybean meal (SBM) + corn + 30 IU E/kg; GOSS = cottonseed meal (CSM) + corn + 30 IU vitamin E/kg; G+VITE = CSM + corn + 4,000 IU vitamin E/animal/d.
[b]As fed basis.
[c]As fed. HPLC procedure (Calhoun et al., 1995; Kim and Calhoun, 1995) done at Texas A&M.
[d]As fed. AOACS procedure. Texas A&M.

Table 4-2. Average composition of dietary supplements[a]

	Supplement		
Item	CONT	GOSS	G+VITE
Average values[c]			
(+)-gossypol (%)	0.0 ± 0.00	0.3 ± 0.03	0.3 ± 0.03
(-)-gossypol (%)	0.0 ± 0.00	0.8 ± 0.08	0.8 ± 0.08
Free gossypol (%)	0.0 ± 0.00	0.1 ± 0.01	0.1 ± 0.01
Total gossypol (%)	0.0 ± 0.00	1.0 ± 0.07	1.0 ± 0.07
Vitamin A (IU/kg)[d]	2076.6	2759.1	2759.1
Vitamin E (IU/kg)[b]	38.8 ± 11.55	44.7 ± 13.11	741.4 ± 182.90
CP(%)[e]	38.6 ± 4.31	37.9 ± 2.03	37.1 ± 2.43
Ca (%)[e]	0.5 ± 0.17	0.5 ± 0.13	0.4 ± 0.12
K (%)[e]	1.4 ± 0.44	1.4 ± 0.10	1.42 ± 0.36
Mg (%)[e]	0.2 ± 0.06	0.6 ± 0.06	0.6 ± 0.10
P (%)[e]	0.7 ± 0.08	1.1 ± 0.05	1.1 ± 0.08
Cu (mg/kg)[e]	16.7 ± 7.65	16.0 ± 2.60	14.9 ± 3.17
Zn (mg/kg)[e]	69.3 ± 28.39	73.3 ± 11.77	70.5 ± 13.85
Mn (mg/kg)[e]	52.6 ± 26.95	36.2 ± 13.51	31.1 ± 13.55
Fe (mg/kg)[e]	182.6 ± 91.51	161.7 ± 76.54	139.0 ± 40.00
Se (mg/kg)[e]	0.2 ± 0.11	0.2 ± 0.04	0.2 ± 0.04

[a]CONT = soybean meal (SBM) + corn + 30 IU E/kg; GOSS = cottonseed meal (CSM) + corn + 30 IU vitamin E/kg; G+VITE = CSM + corn + 4,000 IU vitamin E/animal/d.
[b]As fed basis.
[c]Mean of 8 mixing ± SD.
[d]As fed. A composited sample from all mixing dates. An IU/kg = Retinol acetate (μg/kg) * 2.91
[e]DM basis.

nigrosin-eosin (Society for Theriogenology, Hastings, NE) was prepared. A minimum of 200 sperm were assessed for morphology using oil-immersion bright-field microscopy (x 1000) (Chenoweth et al., 1994). Abnormal sperm were classified based on the region (head, acrosome, midpiece, or tail) where the lesion was present (Chenoweth et al., 1994). Proximal droplets, abnormal acrosomes (e.g., knob), coiled tails, abnormal heads, and abnormal midpieces were categorized as primary sperm abnormalities, and distal cytoplasmatic droplets, kinked tails, and detached heads were categorized as secondary sperm abnormalities.

Animals were sacrificed at 15 months of age using standard industry procedures at the University of Florida Meats Laboratory (Gainesville, FL). Testes were collected and immediately transported on ice to the laboratory. Each spermatic cord was trimmed, and the intact testis and tunic were weighed. The tunic was then removed, and both the tunic and testis were weighed separately. Testicular circumference was measured, and testis length, depth, and width were obtained to determine testicular volume. Paired testicular volume was calculated as the sum of the volume of the right and the left testes. Testes were considered as paraboloids, and volume was calculated using the equation $v = \pi r^2 h$, where r = (width + depth) / 4 and h = length (Chase, et al., 1997). Epididymides were removed, weighed, and mid-parenchymal sections were obtained for in-situ "Tunel" staining, spermatogenesis quantification (5 g), and for ELISA (1 g).

The in-situ "Tunel" staining method used was the ApopTag S7101 kit (Hikim, et al., 1995). This method is based on the specific binding of terminal deoxynucleotidil transferase (TdT) to the newly formed 3'-OH of cleaved DNA. Testicular tissues were

fixed in 10% neutral buffered formalin for 24-36 h and paraffin-embedded and sectioned. Sections were de-paraffinized by two washes with xylene, two washes with absolute ethanol, one wash with 90% ethanol, one with 70% ethanol, and one with DPBS. Endogenous peroxidase was quenched using 2% hydrogen peroxide in DPBS, followed by two rinses with DPBS. Slides were placed in equilibrium buffer and then in working strength TdT enzyme. The reaction was stopped by adding working-strength stop/wash buffer. Two drops of anti-digoxigenin-peroxidase were applied to slides, and the peroxidase was detected with diaminobenzidine. Negative controls were established by adding distilled water instead of TdT enzyme during the preparation of working-strength TdT. Sections were counter-stained with 0.5% methyl green (w:v in 0.1 M sodium acetate, pH 4) and mounted with Permount. Cells stained positive for apoptosis were counted in ten seminiferous tubules epithelium/slide/bull.

Aliquots of homogenized testicular parenchyma in DPBS were used for quantification of spermatogenic apoptosis using a cell death detection ELISA-kit (Hingst and Blottner, 1995). The cell death detection ELISA is a quantitative sandwich-enzyme-immunoassay that uses monoclonal mouse antibodies against DNA and histones that permit determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysetes. Testicular parenchyma (1 g in 2 ml DPBS) was minced, freeze/thawed 3 times, homogenized for 1 min using a Polytron (Brinkmann Instruments, Westbury, NY), sonicated for 1 min at 4° C and then centrifuged at 25000 g for 30 min at 4° C. The supernatant was collected and stored in 100 µl aliquots at -20° C. The microtiterplate-modules (MM) were coated with 100 µl of coating solution containing the anti-histone

antibody, and cultured overnight at 4° C. Incubation buffer 200 µl were pipetted into each well, covered with plate cover foils, and incubated at room temperature (18° to 25° C) for 30 min to saturate the non-specific binding sites of the MM. After incubation, the incubation buffer was removed, and MM were washed three times with 300 µl washing solution. Then 100 µl of each sample solution containing 10 µg of testis equivalent/100 µl were added to the MM, covered, and incubated for 90 min at room temperature. For background determination, 100 µl of incubation buffer were pipetted into three wells. Then, 100 µl of anti-DNA-peroxidase were added to the MM and incubated for 90 min at room temperature. After incubation, ABTS® (Boehringer) substrate was added to the wells, incubated at room temperature for 10 min, and the absorbance read at 405 nm.

Quantitative spermatogenesis was assessed by counting elongated spermatids to determine daily sperm production (DSP) and DSP/g (DSPG) of testicular parenchyma (Chenoweth et al., 1994). Briefly, 5 g of testicular parenchyma were thawed, finely minced, and homogenized for approximately 1 min in 25 ml of working solution (0.9% NaCl, 0.05% Triton X-100, and 100 ppm Merthiolate diluted 1:4 with distilled water). Then, 175 ml of working solution were added, mixed for 1 min, allowed to settle for at least 1 h, and then thoroughly mixed using a stirrer. Numbers of spermatozoa and elongated spermatids were counted using a hemocytometer, and the values were used to determine DSPG and DSP using the formula:

$$DSPG = AX\ (B+Y) / (\text{Time divisor})Y$$

$$DSP = DSPG\ (0.99Z)$$

Where X = hemocytometer count, Y = parenchyma sample weight, Z = testis parenchyma

weight, A = hemocytometer constant, B = dilution factor, and time divisor (5.32 = time divisor for Bos taurus) (Amann et el., 1974).

Statistical Analysis

Data were analyzed by least squares ANOVA, and means were separated by Duncan multiple range test, both using the PROC GLM procedure of SAS (1989). Testicular and semen characteristics as well as spermatogenic apoptosis were analyzed as a completely randomized design. Treatment and pen within treatment effects were tested using bull within pen and treatment as an error term. The pen within treatment effect was removed from the final model because it was not significant ($P \geq 0.2$). All data are presented as least squares means ± SEM (LSMeans ± SEM).

Results

Least squares means (± SEM) for semen and testicular characteristics are shown in Table 4-3. The percentage of normal spermatozoa and the percentage of spermatozoa with primary sperm abnormalities were affected by treatment (30 ± 7.0 vs 68 ± 6.7 and 55 ± 6.0 %, and 59 ± 6.0 vs 24 ± 6.0 and 38 ± 5.2 %, for GOSS, CONT, and G+VITE respectively; $P<0.05$). In contrast, no treatment effect was observed on secondary sperm abnormalities, paired testicular and epididymal weight, or scrotal circumference.

Spermatogenic apoptosis, assessed by Tunel staining (LSMeans ± SEM), is shown in Figure 4-2. The number of apoptotic cells per seminiferous tubule did not differ as result of treatment (0.43 ± 0.05, 0.51 ± 0.05, and 0.52 ± 0.05 for CONT, GOSS, and G+VITE respectively).

Table 4-3. Sperm morphology and testicular traits in young Holstein bulls fed diets containing gossypol, gossypol plus vitamin E, and controls[a]

	Supplement[b]		
Item	CONT	GOSS	G+VITE
Normal, %	68 ± 6.7[d]	30 ± 7.0[e]	55 ± 6.0[d]
Primary abnormalities[c], %	24 ± 6.0[d]	59 ± 6.0[e]	38 ± 5.2[d]
Secondary abnormalities[c], %	8 ± 2.0	11 ± 2.0	6 ± 1.8
Scrotal circumference, cm	32 ± 0.8	31 ± 0.8	31± 0.8
Paired testicular wt, g	495 ± 37.0	490 ± 37.0	564 ± 37.0
Paired testicular vol., cm^3	707 ± 45.0	657 ± 45.0	748 ± 45.0
Paired epididymal wt, g	48 ± 3.1	42 ± 3.1	51± 3.1

[a]Least squares means ± SEM.
[b]CONT = soybean meal (SBM) + corn + 30 IU E/kg; GOSS = cottonseed meal (CSM) + corn + 30 IU vitamin E/kg; G+VITE = CSM + corn + 4,000 IU vitamin E/animal/d.
[c]Primary = proximal droplet, abnormal acrosome, coiled tail, abnormal head, and abnormal midpiece. Secondary = distal cytoplasmic droplet, kinked tail, and detached head.
[d,e]Within rows, LSMeans ± SEM with different superscript are significantly different ($P \leq 0.05$).

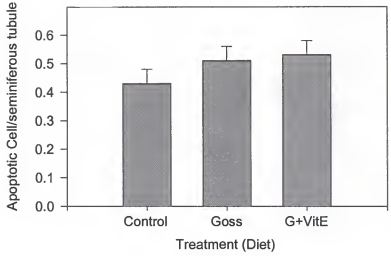

Figure 4-1. Apoptotic cells per seminiferous tubule in bulls fed gossypol (GOSS), gossypol plus vitamin E (G+VITE), and controls (CONT). Spermatogenic apoptosis, assessed by Tunel staining, did not differ between groups.

Sperm production, described as DSPG and DSP (LSMeans ± SEM), is shown in Figure 4-2. Daily sperm production/g (10.2 ± 1.0 vs 14.6 ± 1.0 and 17.6 ± 1.0 $x10^6$ spermatozoa for GOSS, CONT, and G+VITE respectively; $P \leq 0.05$) and DSP (2.2 ± 0.3 vs 3.2 ± 0.3 and 4.1 ± 0.3 $x10^9$ spermatozoa for GOSS, CONT, and G+VITE respectively; $P \leq 0.05$) were lower in gossypol alone (GOSS) fed bulls compared with either control bulls (CONT) or those fed both gossypol and 4000 IU of vitamin E (G+VITE). Least squares means ± SEM for spermatogenic apoptosis in the three groups are also shown in Figure 4-2. Gossypol had no effect on spermatogenic apoptosis (696.9 ± 13.21, 721 ± 14.98, and 703.3 ± 14.01 mU/mg for CONT, GOSS, and G+VITE respectively). However, there was an inverse relationship between sperm production (both DSPG and DSP) and the level of spermatogenic apoptosis ($r=-0.46$; $P<0.05$ and $r=-0.55$; $P<0.05$, DSPG and DSP respectively).

Discussion

Bulls fed diets containing gossypol alone showed a lower percentage of normal spermatozoa and higher percentage of primary sperm abnormalities compared to bulls in CONT and G+VITE groups. Similar results were reported by Chenoweth et al. (1994) where bulls fed similar levels of free gossypol showed a significant reduction in the number of normal spermatozoa and a higher percentage of midpiece abnormalities than did controls. However, in other reports, primary sperm abnormalities were not affected when bulls were fed diets containing gossypol (Jimenez et al., 1989; Chase et al., 1994).

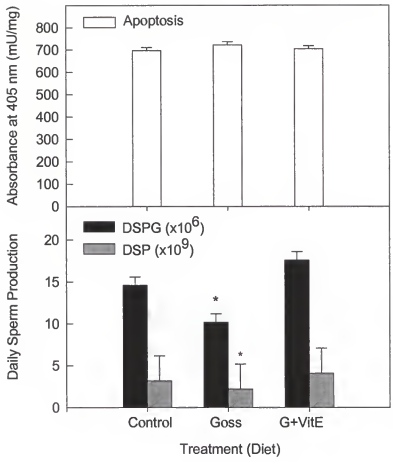

Figure 4-2. Levels of spermatogenic apoptosis and sperm production in bulls fed gossypol (GOSS), gossypol plus vitamin E (G+VITE), and controls (CONT). Spermatogenic apoptosis, assessed by cell death detection ELISA did not differ between groups, but sperm production, assessed as daily sperm production per gram of parenchyma (DSPG) and daily sperm production (DSP), were lower ($P \leq 0.05$) in GOSS than control or G+VITE.

In contrast, in the present study, bulls fed control (CONT) diets and diets containing both gossypol and vitamin E (G+VITE) showed significantly higher levels of normal spermatozoa and lower levels of primary sperm abnormalities than bulls fed gossypol alone (GOSS). This suggests that dietary vitamin E was able to protect the spermatogenic epithelium of bulls from the spermatoxic effect of gossypol.

In the present study, gossypol did not adversely affect testicular traits (paired testicular and epididymal weights, paired testicular volume, and scrotal circumference). However, bulls supplemented with both gossypol and vitamin E showed a tendency to have larger testicular traits (paired testicular and epididymal weights, paired testicular volume, and scrotal circumference) than either control bulls or those fed with gossypol alone. In contrast, bulls supplemented with gossypol alone showed a tendency to have lower testicular traits. A similar tendency of lower, albeit non significant, paired testicular weights have been previously reported for bulls fed gossypol when compared with controls (Chase et al., 1994; Chenoweth et al., 1994), and results in this study are in agreement with previous findings showing that scrotal circumference and testicular weights were not adversely affected by gossypol (Jimenez et al., 1989; Chase et al., 1994).

Daily sperm production (DSP) and daily sperm production per g of testicular parenchyma (DSPG) were lowered in bulls supplemented with gossypol alone (GOSS) than in control (CONT) bulls and those supplemented with gossypol plus vitamin E (G+VITE). Daily sperm production and DSPG in CONT and G+VITE supplemented bulls were within values previously reported for dairy bulls (Amann et al., 1976; Amann,

1981). Lower DSPG and DSP have been previously reported in bulls fed gossypol (Chenoweth et al., 1994). This lower sperm production in GOSS bulls probably occurred via damage to the spermatogenic epithelium, as previously suggested (Randel et al., 1992). Also, since gossypol has been reported to cause damage to the spermatogenic epithelium by reducing the number of germinal cell layers without reducing tubule diameter (Chase et al., 1994), the lack of change in both scrotal circumference and testicular weights in gossypol treated bulls in this study might be expected.

Surprisingly, gossypol treatment alone had no effect on estimated levels of spermatogenic apoptosis (Tunel staining or ELISA), despite significantly reducing sperm production. However, there was a negative correlation between sperm production (DSPG and DSP) and spermatogenic apoptosis. Such a relationship concurs with results obtained during testicular involution in seasonal breeders (Blottner et al., 1995; Hingst and Blottner, 1995), supporting the underlying hypothesis that increased spermatogenic apoptosis is associated with decreased sperm production. Failure to detect differences in spermatogenic apoptosis in this experiment could result from the following considerations; a) the number of bulls studied was too small, b) spermatogenic cells damaged by gossypol, as reflected in decreased spermatogenesis, might undergo a form of cell death different from apoptosis (Young et al., 1997), or c) methods used in this study were inadequate to detect a difference in levels of spermatogenic apoptosis among groups.

In defense of the current procedures, the number of bulls used in this experiment was sufficient to detect significant differences in DSPG and DSP among groups. In

addition, spermatogenic apoptosis has been reported to increase in other species after exposure to hormonal imbalance, spermatoxicity, or increased testicular temperature (Shikone et al., 1994; Richburg and Boekelheide, 1996; Hikim et al., 1997). Also, methods used to assess spermatogenic apoptosis in this study have been previously successfully employed in testicular tissue. The ELISA kit used to quantify spermatogenic apoptosis was similar to one previously used to determine spermatogenic apoptosis in bulls (Hingst and Blottner, 1995). Despite this, spermatogenic apoptosis levels detected in this study were lower than those reported by Hingst and Blottner (1995). The in-situ "Tunel" kit (ApopTag S7101) was similar to one previously used in testicular tissue in the rat (Hikim et al., 1995). Also, in-situ "Tunel" stained slides from our laboratory were validated by Oncor technicians, providing assurance that the methodology and reagents were satisfactory.

In conclusion, free dietary gossypol had an adverse effect on percentage of normal sperm, percentage of primary abnormalities, DSPG, and DSP in young Holstein bulls, although testicular traits and spermatogenic apoptosis were not affected. Vitamin E, fed at 4,000 IU vitamin E/animal/d, was able to counteract the spermatoxic effects of gossypol, suggesting that vitamin E supplementation in the diets of bulls fed cottonseed meal could allow this valuable feedstuffs to be fed safely to puberal bulls.

CHAPTER 5
BREED EFFECTS ON SPERMATOGENESIS AND SPERMATOGENIC APOPTOSIS IN BULLS

Introduction

High environmental temperatures and high humidity, as encountered in subtropical regions of the United States, have been associated with lower fertility of cattle (Dunlap and Vincent, 1971; Badinga et al., 1985). Although high environmental temperatures can cause an increase in early embryonic mortality (Ealy et al., 1993), the bull probably contributes to this problem (Igboeli and Rakha, 1971; Wildeus and Entwistle, 1983), via decreased semen quality during the summer months (Johnston et al., 1963; Igboeli and Rakha, 1971; Fields et al., 1979; Wildeus and Entwistle, 1983; Chase et al., 1993). Lowered semen quality has also been reported to occur after experimental exposure of bulls to elevated temperatures (Johnston et al., 1963; Meyerhoeffer et al., 1985). Breed of sire may also influence both pregnancy rates and calf survival in subtropical environments (Gonzales-Padilla et al., 1969; Crockett et al., 1973). Also, breed differences in semen quality after exposure to elevated temperatures have been reported (Igboeli and Rakha, 1971; Rhynes and Ewing, 1973; Fields et al., 1979; Wildeus and Entwistle, 1983; Chase et al., 1993; Wildeus and Hammond, 1993). Differences in spermatogenic response to elevated temperatures between Bos taurus and Bos indicus

genotypes most likely reflect wider aspects of environmental adaptability which differentiate between these groups (Kumi-Diaka et al., 1981; Kamwanja et al., 1993).

Tropically adapted Bos taurus breeds such as Senepol (N'Dama x Red Poll crossbreed originated in St. Croix, U.S.V.I.) and Romosinuano (criollo-type native of Colombia) have been reported to be heat tolerant (Hammond et al., 1995), as well as Tuli, a Sanga breed (tropically adapted) that originated in Africa. Also, crossbreds such as the Senepol x Angus (SA) and Tuli x Angus (TA) have been reported to be heat tolerant similarly to the Brahman x Angus (BA) (Hammond et al., 1998). Rectal temperatures, during the Florida summer, have been observed to be lower in Senepol and Romosinuano than in Angus bulls (Chase et al., 1993; Hammond 1996). Also, semen quality has been reported to be lower during the summer in Angus and in Holstein bulls when compared with Senepol bulls (Chase et al., 1993; Wildeus and Hammond, 1993). This suggests that spermatogenesis in tropically adapted breeds such as Senepol, Romosinuano, and Brahman could be more efficient than in temperate breeds such as Angus, during periods of elevated ambient temperatures such as during the summer in Florida.

Depression of spermatogenesis following exposure to elevated temperatures has been reported in the bull (Skinner and Louw, 1966; Ross and Entwistle, 1979; Meyerhoeffer et al., 1985; Vogler et al., 1993). Such spermatogenic depression is quite possibly associated with increased spermatogenic apoptosis (or programmed cell death). Evidence to support the idea that spermatogenic apoptosis in the bull could be increased by exposure to elevated temperatures can be found in the rat. In the adult rat testis, spermatogenic apoptosis was reported to increase following elevation of testicular

temperature due to cryptorchidism (Shikone et al., 1994). Spermatogenic apoptosis has been reported to occur in bulls, in seasonal breeders, in hamsters, in rats, in humans, and others (Shikone et al., 1994; Hingst and Blottner, 1995; Hikim et al., 1997; Lin et al., 1997).

The objectives of this study were 1) to evaluate the effect of breed type on testicular traits and extragonadal sperm reserves (ESR) among tropically adapted B. taurus and B. indicus (Romosinuano, Brahman, and Nelore x Brahman) and temperate B. taurus (Angus) bulls; 2) to determine the effect of breed type on testicular and spermatogenic traits and spermatogenic apoptosis in young tropically adapted crossbred bulls during the summer in central Florida (SA, TA, and BA); and 3) to obtain more information on testicular and spermatogenic traits and spermatogenic apoptosis in tropically adapted Miniature Brahman and Romosinuano bulls during the summer in central Florida.

Materials and Methods

Materials

Merthiolate was purchased from Sigma Chemical Co. (St. Louis, MO). Sodium chloride, and triton X-100 were purchased from Fisher Scientific (Fair Lawn, NJ). Dulbeco's phosphate-buffered saline (DPBS) was purchased from Life Technologies Inc. (Grand Island, NY), and cell death detection ELISA-kits were purchased from Boehringer Mannheim Co. (Indianapolis, IN).

Experiment 1

Young purebred (20 months of age; n=36; Angus, An, temperate B. taurus, n=9; Romosinuano, Ro, tropically adapted B. taurus, n=9; Brahman, Br, B. indicus, n=9; Nelore x Brahman, NB, B. indicus, n=9) bulls were evaluated for testicular and spermatogenic traits and extragonadal sperm reserves at the Subtropical Agricultural Research Station, Brooksville, Florida (28° 37' N latitude, 82° 22' W longitude) during the summer. Animals were sacrificed using standard industry procedures at Central Packing Inc. (Center Hill, Florida). Testes were collected and immediately transported on ice to the laboratory. Each spermatic cord was trimmed, and the intact testis and tunic were weighed. The tunic was then removed, and both the tunic and the testis without tunic were weighed separately. Testicular circumference was measured, and testis length, depth, and width were obtained to determine testicular volume. Paired testicular volume was calculated as the sum of the volume of the right and the left testes. Testes were considered paraboloids, and the volume was calculated using the equation $v = \pi r^2 h$, where r = (width + depth) / 4 and h = length (Chase, et al., 1997). Epididymides were removed, weighed and mid-parenchymal sections were obtained for spermatogenesis quantification (5 g).

Quantitative spermatogenesis was assessed by counting elongated spermatids to determine daily sperm production (DSP) and DSP/g of testicular parenchyma (DSPG) (Chenoweth et al., 1994). Briefly, 5 g of testicular parenchyma were thawed, finely minced, and homogenized for approximately 1 min with 25 ml of working solution (0.9% NaCl, 0.05% Triton X-100, and 100 ppm Merthiolate diluted 1:4 with distilled water).

Then, 175 ml of working solution were added, mixed for 1 min, allowed to settle for at least 1 h, and then thoroughly mixed using a stirrer. Number of spermatozoa and elongated spermatids were counted using a hemocytometer, and the values were used to determine DSPG and DSP using the formula:

$$DSPG = AX (B+Y) / (Time\ divisor)Y$$

$$DSP = DSPG (0.99Z)$$

Where X = hemocytometer count, Y = parenchyma sample weight, Z = testis parenchyma weight, A = hemocytometer constant, B = dilution factor, and time divisor (TD) (5.32 = TD for Bos taurus, An and Ro; and 5.11 = TD for Bos indicus, Br and NB) (Amann et al., 1974; Chenoweth et al., 1994). For extragonadal sperm reserves, the right epididymis was separated into caput (head), corpus (body), and cauda (tail). Sperm reserves were calculated using the following formula:

$$ESR = AX (B+W)$$

Where A = hemocytometer constant, X = hemocytometer count, B = dilution factor, and W = weight of relevant segment of epididymis (head, body or tail) (Wildeus, 1993).

Experiment 2

Tropically adapted young crossbred bulls (n=112; averaging 17 months of age; Senepol x Angus, SA; Tuli x Angus, TA; Brahman x Angus, BA) were evaluated for testicular and spermatogenic traits (as described for experiment 1) and spermatogenic apoptosis at the Subtropical Agricultural Research Station, Brooksville, Florida. The experiment was repeated over two years using 58 (SA, n=18; TA, n=22; BA, n=18) bulls in year 1 and 54 (SA, n=22; TA, n=18; BA, n=14) bulls in year 2.

Daily sperm production (DSP) and DSPG and in BA were calculated by using both the TD for Bos taurus and for Bos indicus (5.32 and 5.11 respectively), whereas only the TD for Bos taurus was employed for the other two breeds (SA and TA).

Aliquots of homogenized testicular parenchyma in DPBS were used for quantification of spermatogenic apoptosis using a cell death detection ELISA-kit (Hingst and Blottner, 1995) in a subset of 10 bulls/breed/year (n=60). The cell death detection ELISA is a quantitative sandwich-enzyme-immunoassay that uses monoclonal mouse antibodies against DNA and histones that allows determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. Testicular parenchyma (1 g in 2 ml DPBS) was minced, freeze/thawed 3 times, homogenized for 1 min using a Polytron (Brinkmann Instruments, Westbury, NY), sonicated for 1 min at 4° C and then centrifugation at 25000 g for 30 min at 4° C. The supernatant was collected and stored in 100 µl aliquots at -20° C. The microtiterplate-modules (MM) were coated with 100 µl of coating solution containing the anti-histone antibody, and cultured overnight at 4°C. Incubation buffer 200 µl were pipetted into each well, covered with plate cover foils, and incubated at room temperature (18° to 25° C) for 30 min to saturate the non-specific binding sites of the MM. After incubation, the incubation buffer was removed, and MM were washed three times with 300 µl washing solution. Then 100 µl of each sample solution containing 10 µg of testis equivalent/100 µl were added to the MM, covered, and incubated for 90 min at room temperature. For background determination, 100 µl of incubation buffer were pipetted into three wells.

Then, 100 µl of anti-DNA-peroxidase were added to the MM and incubated for 90 min at room temperature. After incubation, ABTS® (Boehringer) substrate was added to the wells, incubated at room temperature for 10 min, and the absorbance read at 405 nm.

Experiment 3

A third group of 7 Brahman (Brahman, n=2 and miniature Brahman, n=5, averaging 31 and 52 months of age respectively) bulls as well as 6 Romosinuano (19 months of age) bulls were analyzed for testicular and spermatogenic traits (DSP and DSPG, using a TD=5.11 for Brahman and miniature Brahman and a TD=5.32 for Romosinuano) and spermatogenic apoptosis during the summer in Florida as described for previous experiments .

Statistical Analysis

Data for experiment 1, 2, and Brahman and miniature Brahman were analyzed by least squares ANOVA using SAS (1989). In experiment 1, the model included breed, and the residual was used as the error term. In experiment 2, the model included year, breed, and breed by year. Data was analyzed with and without correcting for body weight, and the residual was used as the error term. Preplanned orthogonal contrasts (BA vs SA and TA, SA vs TA) were used to compare the effect of breed. In experiment 3, Brahman and miniature Brahman model included breed and the residual was used as the error term. For Romosinuano, means and standard error of the mean were calculated for testicular and spermatogenic traits as well as for spermatogenic apoptosis.

Results

Experiment 1

Angus and Br bulls were older at sacrifice than Ro and NB ($P \leq 0.05$) Table 5-1. Body weight and testicular traits (LSMeans ± SEM for testicular circumference, paired testicular volume, paired testicular weight, and paired epididymal weight) in B. taurus and B. indicus bulls (An, Br, Ro, NB) are shown in Table 5-1. Paired testicular volume and weight as well as right epididymal head weight were greater in An bulls when compared to Br, Ro, and NB ($P \leq 0.05$).

Least squares means for spermatogenesis (DSP and DSPG) and extragonadal sperm reserves (caput, corpus, and cauda epididymis) in young B. taurus and B. indicus bulls (An, Br, Ro, and NB) are shown in Table 5-2. There was no effect of breed on DSP, DSPG, ESR (caput), and ESR (cauda). However, ESR (corpus) in Br was higher than for An and Ro (3.1 ± 0.54 vs 1.2 ± 0.55 and 1.7 ± 0.57 x10^9 spermatozoa respectively; $P \leq 0.05$).

Experiment 2

Least squares means (±SEM) for age, body weight, and testicular traits (paired testicular volume, paired testicular and epididymal weight and testicular circumference) in crossbred (BA, SA, and TA) bulls pooled for both years are shown in Table 5-3. No difference was found in age among breeds, but BA were heavier at sacrifice than SA and TA bulls (485.0 ± 8.68 vs 427.5 ± 7.74 and 427.8 ± 7.74 kg; $P \leq 0.05$).

Table 5-1. Physical and testicular traits in young Bos taurus and Bos indicus bulls[a].

Item	Breed			
	Angus	Brahman	Romosinuano	Nelore x Brahman
No. of bulls	8	8	8	8
Age, days[b, c]	662.4 ± 8.11	658.6 ± 8.11	630.7 ± 8.11	634.7 ± 8.11
Body Weight, kg	489.4 ± 13.23	466.7 ± 13.23	457.6 ± 13.23	473.5 ± 13.23
Testicular circumference, cm	18.0 ± 0.72	17.6 ± 0.67	18.1 ± 0.70	18.1 ± 0.68
Paired testicular vol[d], cm^3	765.3 ± 63.29	600.5 ± 54.47	602.6 ± 53.59	570.8 ± 54.85
Paired testicular wt[d], g	489.6 ± 34.06	388.1 ± 31.72	404.3 ± 33.72	434.0 ±32.24
Paired epididymal wt, g	50.1 ± 3.11	45.5 ± 2.90	46.4 ± 3.03	45.5 ± 2.94

[a]Least squares means ± SEM.
[b]Contrast, Romosinuano vs Angus and Brahman ($P \leq 0.05$).
[c]Contrast, Nelore x Brahman vs Angus and Brahman ($P \leq 0.05$).
[d]Contrast, Angus vs Brahman, Romosinuano, and Nelore x Brahman ($P \leq 0.05$).

Table 5-2. Spermatogenic traits and extragonadal sperm reserves in young Bos taurus and Bos indicus bulls[a].

Item	Breed			
	Angus	Brahman	Romosinuano	Nelore x Brahman
No. of bulls	8	8	8	8
Spermatogenesis				
DSP[b] (x 10^9)	5.3 ± 0.58	3.7 ± 0.55	5.4 ± 1.73	4.5 ± 0.53
DSPG[c] (x 10^6)	21.9 ± 2.02	19.7 ± 1.94	24.1 ± 6.07	21.2 ± 1.87
Epididymal Extragonadal sperm reserves				
Caput (x 10^9)	4.9 ± 0.89	4.5 ± 0.93	3.3 ± 0.90	3.7 ± 0.84
Corpus (x 10^9)[d, e]	1.2 ± 0.55	3.1 ± 0.54	1.7 ± 0.57	2.6 ± 0.55
Cauda (x 10^9)	2.8 ± 0.76	2.4 ± 0.79	3.7 ± 0.77	3.1 ± 0.72

[a]Least squares means ± SEM.
[b]Daily sperm production, DSP (x 10^9).
[c]Daily sperm production per gram of testicular parenchyma DSPG (x 10^6).
[d]Contrast, Brahman vs Angus and Romosinuano ($P \leq 0.05$).
[e]Contrast, Nelore x Brahman vs Angus ($P \leq 0.05$).

Table 5-3. Physical and testicular traits in young crossbred bulls[a].

	Breed		
Item	Brahman x Angus	Senepol x Angus	Tuli x Angus
No. of bulls	32	40	40
Age, days	496 ± 8.7	511 ± 7.8	518 ± 7.8
Body weight[e], kg	485 ± 8.7	428 ± 7.7	428 ± 7.7
Testicular circumference[b,c,e], cm	17.4 ± 0.22	17.9 ± 0.18	18.2 ± 0.18
Testicular circumference[d], cm	18.1 ± 0.25	17.6 ± 0.22	17.9 ± 0.22
Paired testicular vol[b,c,e], cm^3	720 ± 27.0	734 ± 21.8	777 ± 21.8
Paired testicular vol[d,e], cm^3	799 ± 28.9	705 ± 25.5	748 ± 25.5
Paired testicular wt[b,c,e], g	447 ± 16.6	473 ± 13.7	489 ± 13.6
Paired testicular wt[d], g	499 ± 18.5	453 ± 16.5	469 ± 16.5
Paired epididymal wt[b,c,e], g	51.5 ± 1.65	52.5 ± 1.36	52.9 ± 1.36
Paired epididymal wt[d,e], g	55.1 ± 1.67	51.1 ± 1.49	51.5 ± 1.49

[a]Least squares means ± SEM.
[b]Adjusted for body weight.
[c]Adjusted for body weight ($P \leq 0.01$).
[d]Unadjusted data.
[e]Orthogonal contrast, Brahman x Angus vs Senepol x Angus and Tuli x Angus ($P \leq 0.05$).

Testicular circumference was lower in BA than in SA and TA (17.4 ± 0.22 vs 17.9 ± 0.18 and 18.2 ± 0.18 cm; P≤ 0.05). However, breed type did not affect other testicular traits evaluated. There was an effect of body weight on testiticular traits (P< 0.01).

Least squares means (±SEM) for spermatogenic traits (DSP and DSPG) in crossbred bulls are shown in Table 5-4. When spermatogenesis was calculated using a TD for B. taurus (5.32), both DSP and DSPG were lower for BA in comparison with SA and TA (5.4 ± 0.38 vs 6.2 ± 0.31 and 6.6 ± 0.31 x 10^6; P≤0.05 and 1.2 ± 0.12 vs 1.5 ± 0.10 and 1.6 ± 0.10 x 10^9; P≤0.05, respectively). In contrast, when spermatogenesis for BA alone was calculated using a TD for B. indicus (5.11), only DSP was lower for BA when compared with SA and TA (1.3 ± 0.12 vs 1.5 ± 0.10 and 1.6 ± 0.10 x 10^9; P≤0.05, respectively). In both cases, DSP was affected by body weight (P< 0.01).

Results of estimates of spermatogenic apoptosis, as measured by cell death detection ELISA, in crossbred bulls are shown in Figure 5-1. There was no effect of breed on spermatogenic apoptosis when BA, SA, and TA were compared (1264 ± 179, 1039.5 ± 184.23, and 917.6 ± 179.32 mU/mg of testicular parenchyma, respectively).

Experiment 3

Least squares means (± SEM) for testicular traits, spermatogenesis, and spermatogenic apoptosis in Brahman and Miniature Brahman bulls are shown in Table 5-5. Paired testicular volume, paired testicular weight, paired epididymal weight, and DSP were smaller in miniature Brahman than in Brahman bulls (P≤0.05).

Table 5-4. Spermatogenic traits and spermatogenic apoptosis in young crossbred bulls[a].

	Breed		
Item	Brahman x Angus	Senepol x Angus	Tuli x Angus
No. of bulls	32	40	40
Time divisor	5.32	5.32	5.32
DSP[b,d,e,g] (x 10^9)	1.2 ± 0.12	1.5 ± 0.10	1.6 ± 0.10
DSP[b,f] (x 10^9)	1.4 ± 0.11	1.5 ± 0.10	1.6 ± 0.10
DSPG[c,d,g] (x 10^6)	5.4 ± 0.38	6.2 ± 0.31	6.6 ± 0.31
DSPG[c,f,g] (x 10^6)	5.5 ± 0.34	6.1 ± 0.31	6.6 ± 0.31
Time divisor	5.11	5.32	5.32
DSP[b,d,e,g] (x 10^9)	1.3 ± 0.12	1.5 ± 0.10	1.6 ± 0.10
DSP[b,f] (x 10^9)	1.5 ± 0.11	1.5 ± 0.10	1.6 ± 0.10
DSPG[c,d] (x 10^6)	5.6 ± 0.39	6.2 ± 0.31	6.6 ± 0.31
DSPG[c,f] (x 10^6)	5.7 ± 0.35	6.1 ± 0.31	6.6 ± 0.31
No. of bulls	20	20	20
Spermatogenic apoptosis, mU/mg	1264 ± 179.0	1040 ± 184.2	918 ± 179.3

[a]Least squares means ± SEM.
[b]Total daily sperm production (DSP x 10^9).
[c]Daily sperm production per gram of testicular parenchyma (DSPG x 10^6).
[d]Adjusted for body weight.
[e]Adjusted for body weight ($P \leq 0.01$).
[f]Unadjusted data.
[g]Orthogonal contrast, Brahman x Angus vs Senepol x Angus and Tuli x Angus ($P \leq 0.05$).

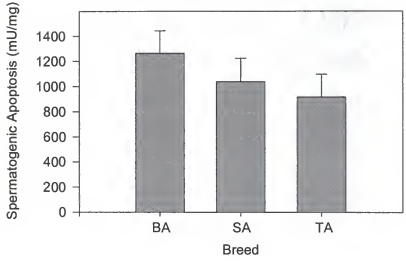

Figure 5-1. Least squares means (± SEM) for spermatogenic apoptosis in crossbred bulls (BA= Brahman x Angus SA= Senepol x Angus, and TA= Tuli x Angus).

Table 5-5. Testicular traits, spermatogenesis, and spermatogenic apoptosis in Brahman and miniature Brahman bulls[a].

	Breed	
Item	Brahman	Miniature Brahman
No. of bulls	2	5
Age, days	941.5 ± 694.84	1573.4 ± 439.46
Testicular circumference, cm	15.1 ± 1.15	15.3 ± 0.94
Paired testicular vol.[b], cm^3	557.9 ± 15.07	402.6 ± 12.30
Paired testicular wt[b], g	437.5 ± 11.56	278.8 ± 9.44
Paired epididymal wt[b], g	57.6 ± 3.35	44.0 ± 2.73
DSP[c,b] (x 10^9)	2.7 ± 0.16	1.5 ± 0.13
DSPG[d] (x 10^6)	12.0 ± 1.90	9.9 ± 1.20
Spermatogenic apoptosis, mU/mg	2779.8 ± 140.29	2592.1 ± 88.72

[a]Least squares Means ± SEM.
[b]Brahman was different from Miniature Brahman ($P \leq 0.05$).
[c]Daily sperm production, DSP (x 10^9).
[d]Daily sperm production per gram, DSPG (x 10^6).

In contrast, testicular circumference, DSPG, and spermatogenic apoptosis did not differ among breeds. Means ± SEM for testicular traits, spermatogenesis, and spermatogenic apoptosis in Romosinuano bulls are shown in table 5-6. Romosinuano bulls in experiment 3 were 67 d younger than Ro bulls in experiment 1. Romosinuano bulls in experiment 3 showed similar testicular traits (paired testicular volume, paired testicular weight, paired epididymal weight, and testicular circumference) when compared with the Ro bulls in experiment 1, although DSP and DSPG were less in Ro in experiment 3 than Ro bulls in experiment 1.

Discussion

A comparison of testicular traits, spermatogenesis, and extragonadal sperm reserves in 20 mo Angus, Romosinuano, Brahman, and Nelore x Brahman bulls showed differences in paired testicular volume and paired testicular weight However, quantitative spermatogenic traits (DSP and DSPG) did not differ among temperate (Angus) and tropically adapted (Romosinuano, Brahman, and Nelore x Brahman) breeds. Extragonadal sperm reserves were not different among breeds with the exception of those in the ESR (corpus). When tropically adapted crossbreeds were compared for testicular traits, spermatogenesis, and spermatogenic apoptosis, results showed differences in testicular circumference and possibly spermatogenesis. However, spermatogenic apoptosis was not different among crossbreeds.

Table 5-6. Testicular traits, spermatogenesis, and spermatogenic apoptosis in Romosinuano bulls[a].

	Breed
Item	Romosinuano
No. of bulls	6
Age, days	563.0 ± 4.22
Testicular circumference, cm	17.5 ± 0.46
Paired testicular vol., cm^3	652.6 ± 46.92
Paired testicular wt, g	426.7 ± 24.63
Paired epididymal wt, g	49.6 ± 2.35
DSP[b] (x 10^9)	2.7 ± 0.32
DSPG[c] (x 10^6)	12.3 ± 1.06
Spermatogenic apoptosis, mU/mg	1730.6 ± 193.16

[a]Means ± SEM.
[b]Daily sperm production, DSP (x 10^9).
[c]Daily sperm production per gram SPG (x 10^6).

In experiment 1, An and Br bulls were older than Ro and NB by approximately 1 month. Paired testicular volume and paired testicular weight were larger in An than in Br, Ro, and NB. In contrast, BW, testicular circumference, and paired epididymal weight were not different among breeds. Angus and Ro (B. taurus) had higher DSP than those reported for yearling Angus and Hereford bulls in Colorado which had similar testicular weights (Berndtson and Igboeli, 1989). However, contrary to the hypothesized results for this study, spermatogenic efficiency (DSPG) did not differ among temperate and tropically adapted breeds. As expected, paired testicular weight, ESR (caput), and ESR (cauda) in this study, were lower in An and Ro (20 mo of age) than in 7 year old Angus and Hereford bulls (Weisgold and Almquist, 1979). Also, testicular traits, DSP, DSPG, and ESR for An and Ro (temperate and tropically adapted B. taurus, respectively) were lower than those reported for B. taurus dairy bulls (Amann et al., 1976). However, DSP and DSPG were higher, and ESR (corpus) in the present study were similar when compared to the results reported by Weisgold and Almquist (1979).

In the present study, no difference was found between Br and NB in any of the parameters studied. Paired testicular and epididymal weights were smaller for Br and NB than those reported for Brahman bulls of similar age and body weights in Florida and Texas (Chenoweth et al., 1994; Chase et al., 1994). In contrast, paired testicular weight was similar to that reported for B. indicus strains in north Australia by Wildeus and Entwistle (1982), whereas paired testicular volume was greater for Br and NB in this experiment in comparison with previous results from Brahman bulls in Texas (Chase et al., 1994).

Daily sperm production (that is influenced by total testicular parenchyma) was less in Br and NB bulls than those reported for Brahman bulls in Florida (Chenoweth et al., 1994), but greater than those reported for Brahman bulls in Texas (Rocha et al., 1996) and B. indicus bulls in tropical Australia (Wildeus and Entwistle, 1982). However, DSPG (reflecting spermatogenic efficiency) was greater in Br and NB bulls in this study than those reported for B. indicus bulls in Florida, Texas, and Tropical Australia (Wildeus and Entwistle, 1982; Chenoweth et al., 1994; Rocha et al., 1996). Extragonadal sperm reserves (Caput) and ESR (corpus) for Br and NB were larger in our study as compared with previous reports for B. indicus bulls (Wildeus and Entwistle, 1982; Rocha et al., 1996). Extragonadal sperm reserves (cauda) was larger in the present study than those reported by Rocha et al. (1996) but smaller than those reported by Wildeus and Entwistle (1982). These results are in agreement with a previous report where breed of bull did not influence extragonadal sperm reserves (Coulter et al., 1987). However, in another study, extragonadal sperm reserves have been reported to be influenced by breed and diet (Coulter and Kozub, 1984).

In experiment 2, although BA bulls were heavier than SA and TA at 508 d of age, their testicular circumference was smaller. These results are in agreement with previous reports in bulls of similar age. For example, when B. indicus bulls were compared for development with B. taurus (from 269 to 619 d of age), B. indicus bulls were heavier than B. taurus at equivalent ages, but scrotal circumference was smaller (Chase et al., 1997). This could be due to slower development of scrotal circumference in B. indicus breeds when compared to B. taurus (Chenoweth, et al., 1996). Body weight at 508 d of

age for these tropically adapted crossbreeds were similar for those reported for 3 year old B. indicus x B. taurus crossbred bulls in Australia (Wildeus and Entwistle, 1983). Paired testicular weight and paired epididymal weight were greater in all crossbreds evaluated in this study than those reported for 3 year old B. indicus x B. taurus crossbred bulls in Australia (Wildeus and Entwistle, 1983), whereas DSP and DSPG values were higher in B. indicus x B. taurus crossbred bulls reported by Wildeus and Entwistle (1983).

In the present study, testicular circumference, paired testicular volume, paired testicular weight, and paired epididymal weight did not differ among breeds, and were of similar magnitude for the same traits reported for Brahman (637 d of age) bulls in another study (Chase et al., 1994).

Daily sperm production/g of testicular parenchyma (DSPG) refers to spermatogenic efficiency, whereas DSP is proportional to the total sperm production of the total testicular parenchyma. In this study, DSP and DSPG were lower for BA than SA and TA when a TD for B. taurus was used. In contrast when a B. indicus TD was used to calculate DSP and DSPG for BA only, DSP was lower in BA when compared to SA and TA. Daily sperm production/g was similar among breed, indicating that spermatogenic efficiency was similar among breeds. However, DSP (and perhaps DSPG) were lower in BA bulls (depending on TD used), suggesting that the BA bulls might have had less testicular parenchyma than either SA or TA bulls. This was reinforced in this experiment where BA had smaller testicular circumferences than the other breed types. Another possible explanation for the difference in spermatogenic traits observed for BA bulls when compared to SA and TA, could be the B. indicus genetic component of BA, since it

has been reported that B. indicus originated bulls reached puberty later than B. taurus and had slower testicular development subsequently than B. taurus breeds (Stewart et al., 1980; Fields et al., 1982; Chenoweth et al., 1996; Chase et al., 1997). Daily sperm production/g and DSP for bulls in experiment 2 were considered to be within values reported for B. indicus strain bulls of similar development in Australia (Wildeus and Entwistle, 1982), but lower than those reported for yearling Hereford and Angus bulls (Coulter et al., 1987; Berndtson and Igboeli, 1989). Also, DSP and DSPG were both lower than those reported for Brahman crossbred, Bali cattle (B. sondaicus), and hybrid bulls ranging from 3 to 7 years of age (N'Dama et al., 1983; Wildeus and Entwistle, 1983; Cardoso and Godinho, 1985; McCool, 1990; Tegegne et al., 1992).

In experiment 3, the testicular traits of Brahman bulls were within the ranges reported for Brahman bulls in Florida (Chenoweth et al., 1994). Miniature Brahman were smaller than Brahman bulls, and the difference in testicular traits (paired testicular volume, paired testicular weight, and paired epididymal weight) are probably as a result of smaller body size. However, testicular circumference did not differ between Brahman and miniature Brahman despite 40% difference in testicular weight. Daily sperm production was lower in miniature Brahman than in Brahman, because DSP is influenced by total testicular parenchyma, and the testes were smaller in miniature Brahman than in Brahman bulls. In contrast, DSPG (spermatogenic efficiency) was not different among the two breeds, and since spermatogenic efficiency did not differ, spermatogenic apoptosis was also not expected to be different. This indicates that the rate of cellular

damage within the spermatogenic epithelium caused by summer elevated temperatures in Florida is similar among miniature Brahman and Brahman bulls.

In conclusion, when temperate B. taurus and tropically adapted B. taurus and B. indicus bulls of similar age and raised under the same conditions were compared, An bulls were heavier and some testicular traits were greater than in tropically adapted bulls (Br, Ro, and NB). However, spermatogenesis and extragonadal sperm reserves did not differ among breeds, with the exception of ESR (corpus) in Br bulls which was greater than in An and Ro bulls. Testicular and spermatogenic traits, including levels of spermatogenic apoptosis, in tropically adapted crossbred breeds used in the present study (i.e. SA, TA, and BA), were not different when assessed during the Florida summer. In contrast, DSP (and perhaps DSPG) were lower in BA than in SA and TA bulls in experiment 2. Here, as it is assumed that DSPG was similar among breeds, this would indicate that spermatogenic efficiency did not differ among breeds, and the difference in DSP was most probably related to differences in the amount of testicular parenchyma as reflected in testicular size. Also, more information regarding testicular traits, spermatogenesis, and spermatogenic apoptosis was obtained for miniature Brahman as compared with Brahman bulls, as well as for the Romosinuano (B. taurus) bulls during the summer in Florida. Brahman and miniature Brahman bulls were older than the rest of bulls used in this series of experiments, and their levels of spermatogenic apoptosis were higher than levels in younger bulls. Then, it is possible to speculate that age could influence the levels of spermatogenic apoptosis, resulting in higher levels of spermatogenic apoptosis in older than in younger bulls.

CHAPTER 6
SUMMARY AND CONCLUSIONS

The effect of elevated temperatures and gossypol toxicity on spermatogenic apoptosis, testicular traits, and the use of antioxidants to counter the effects of gossypol in bulls were studied, as well as breed differences (B. indicus and B. taurus, tropically adapted vs B. taurus temperate) in response to elevated temperatures.

In Chapter 3, two experiments were designed to induce and assess spermatogenic apoptosis. Experiment 1, tested the hypothesis that spermatogenic stress, caused by elevation of body temperature, by E. coli endotoxin or elevation of testicular temperature, by scrotal insulation, increases spermatogenic apoptosis. In this experiment three groups were included; 1) control (n=3), 2) E. coli endotoxin (100 ng/kg) (n=4), and 3) scrotal insulation for 48 h (n=4). Bulls were sacrificed 10 days after initiation of treatment. Results of experiment 1, showed an effect of time of assessment on respiration rate and rectal temperature($P<0.05$ respectively). However, there was no apparent effect of treatment on testicular and semen traits, spermatogenic traits determined by DSP and DSPG, and level of spermatogenic apoptosis.

In experiment 2, the hypothesis that SI increased spermatogenic apoptosis was tested, with additional objectives being to test breed differences in spermatogenic apoptosis and changes in levels of spermatogenic apoptosis with respect to time after SI.

This experiment included two groups; 1) control (Angus, temperate B. taurus, n = 4; Senepol, tropically adapted B. taurus, n = 2; Romosinuano, tropically adapted B. taurus, n = 2) and 2) scrotal insulation (SI) for 48 h (Angus, n = 4; Senepol, n = 4; Romosinuano, n = 2). Bulls were sacrificed at either 2 d or 4 d after initiation of treatments. In experiment 2, the body weight of Angus bulls was significantly lower than Senepol and Romosinuano ($P< 0.001$), whereas rectal and scrotal temperatures were higher in Angus when compared to Senepol and Romosinuano bulls ($P<0.05$). However, rectal temperature was not affected by scrotal insulation. Scrotal temperature was not affected by breed, and the temperature of the scrotum was higher in scrotal insulated after 24 h than in control bulls ($P<0.01$). Semen characteristics and paired epididymal weights were not affected by 48 h of scrotal insulation or breed. Scrotal circumference, paired testicular volume, testicular circumference, and paired testicular weight were smaller in Angus bulls than Senepol and Romosinuano ($P<0.001$ respectively). Scrotal insulation had no effect on DSPG and spermatogenic apoptosis, although DSP was significantly lower in Angus than in Senepol and Romosinuano bulls ($P<0.01$). This suggested that spermatogenic efficiency was not different between breeds and the difference in DSP was due to differences in testicular size.

Experiments designed to induce and assess spermatogenic apoptosis in Chapter 3 failed to detect increases in spermatogenic apoptosis as assessed by histology, tunel stain, and cell death detection ELISA in bulls after E. coli endotoxin infusion or 48 h of scrotal insulation at 2 d, 4 d, or 10 d after initiation of treatment. This suggested that either these treatments caused no spermatogenic apoptosis changes or that the detection windows

used were inappropriate, since no other signs of cell death were observed when evaluated by histology. Also, these results suggest that the levels of spermatogenic apoptosis that were observed, correspond to basal levels of spermatogenic apoptosis, resulting probably from spontaneous degeneration of germ cells, that undergo apoptosis during normal spermatogenesis, (Blanco-Rodriguez and Martinez-Garcia, 1996). It is clear that more work should be done in bulls to elucidate the role of spermatogenic apoptosis in spermatogenic dysfunction.

It has been suggested that the deleterious effect of elevated temperatures on spermatogenesis occurs through an imbalance or excess of reactive oxygen species (Loven et al., 1988). In Chapter 4, an experiment was designed to test the effects of long term feeding gossypol on semen traits, testicular traits, spermatogenesis, and spermatogenic apoptosis with provision to test this theory via use of a dietary antioxidant (vitamin E) in an attempt to counteract spermatoxic effects of gossypol in the bull (Velasquez-Pereira, 1995). Gossypol is a toxic phenolic pigment found in the cotton plant, and cotton products containing gossypol such as cottonseed meal are commonly used as feedstuffs for cattle. In this experiment young Holstein bulls (n=24; 6 mo of age) were distributed into three experimental groups (each n=8); 1) CONT, supplemented with soybean meal (SBM), corn, and 30 IU of vitamin E/kg, 2) GOSS, supplemented with cotton seed meal (CSM), corn, and 30 IU vitamin E/kg, and 3) G+VITE, supplemented with, corn, and 4,000 IU vitamin E/bull/d. Supplements GOSS and G+VITE were formulated to supply 14 mg of free gossypol/kg body weight (BW)/d, and bulls were supplemented from 6 to 15 mo of age.

Results showed that the percentage of normal spermatozoa was lower and percentage of primary sperm abnormalities was higher in the GOSS supplemented bulls when compared to CONT or G+VITE ($P<0.05$). In contrast, no treatment effect was observed on secondary sperm abnormalities, paired testicular and epididymal weight, and scrotal circumference. Spermatogenesis, described in terms of DSP and DSPG, was lower ($P \leq 0.05$) in gossypol fed bulls compared with either control bulls (CONT) or those fed both gossypol and 4000 IU of vitamin E (G+VITE). Surprisingly, gossypol had no effect on spermatogenic apoptosis, despite significantly affecting sperm production, although there was an inverse relationship between sperm production (both DSP and DSPG) and the level of spermatogenic apoptosis ($r=-0.55$; $P<0.05$ and $r=-0.46$; $P<0.05$, for DSP and DSPG respectively). Less than 1 apoptotic cell per seminiferous tubule was observed (tunel stain or histology) in the spermatogenic epithelium in all three groups (CONT, GOSS, and G+VITE).

Free gossypol had an adverse effect on percentage of normal sperm, percentage of primary abnormalities, DSP, and DSPG, although testicular measures and spermatogenic apoptosis were not affected. Vitamin E fed at 4,000 IU vitamin E/animal/d was able to block the spermatoxic effects of gossypol on spermatogenesis, suggesting that vitamin E supplementation in the diets of bulls fed with cottonseed meal could allow this valuable feedstuff to be fed safely to intact bulls.

In Chapter 5, the relative susceptibility of B. taurus and B. indicus and crossbred bulls to environmentally elevated temperature effects on testicular and spermatogenic traits and spermatogenic apoptosis were evaluated during the summer in central Florida.

Two experiments were designed to test the hypothesis that tropically adapted B. taurus and B. indicus breeds will have better semen and testicular traits and lower spermatogenic apoptosis than a temperate breed (Angus) during the summer. In experiment 1, young B. taurus and B. indicus bulls (20 months of age; n=36; Angus, temperate B. taurus, An, n=9; Romosinuano, tropically adapted B. taurus, Ro, n=9; Brahman, tropically adapted B. indicus, Br, n=9; Nelore x Brahman, tropically adapted B. indicus, NB, n=9) were evaluated for testicular and spermatogenic traits and extragonadal sperm reserves at the Subtropical Agricultural Research Station, (28° 37' N latitude, 82° 22' W longitude), Brooksville, Florida. Results for this experiment showed that paired testicular volume and paired testicular weight were greater in An bulls when compared to Br, Ro, and NB ($P \leq 0.05$). There was no effect of breed on DSP, DSPG, ESR (caput), and ESR (cauda). However, ESR (corpus) in Br was higher than An and Ro ($P \leq 0.05$).

In experiment 2, the objective was to compare tropically adapted B. taurus crossbred (Senepol x Angus and Tuli x Angus) with tropically adapted B. taurus x B. indicus crossbred (Brahman x Angus). Young crossbred (17 months of age; n=112; Senepol x Angus, SA; Tuli x Angus, TA; Brahman x Angus, BA) bulls were evaluated in June for testicular and spermatogenic traits and spermatogenic apoptosis at the Subtropical Agricultural Research Station, Brooksville, Florida. The experiment was performed twice using 58 (SA, n=18; TA, n=22; BA, n=18) bulls in year 1 and 54 (SA, n=22; TA, n=18; BA, n=14) in year 2. Results showed that bull ages were similar. Brahman x Angus bulls were heavier at sacrifice than SA and TA bulls ($P<0.05$). Testicular circumference was lower in BA than in SA and TA ($P \leq 0.05$). However, breed

did not affect other testicular traits evaluated. When spermatogenesis was calculated using a TD for B. taurus (5.32), DSP and DSPG for BA were lower than for SA and TA ($P \leq 0.05$). In contrast, when spermatogenesis for BA was calculated using a TD for B. indicus (5.11) while the other breeds used the B. taurus TD, DSP was significantly lower when compared to SA and TA ($P \leq 0.05$). In this experiment, DSP and perhaps DSPG were lower in BA than SA and TA bulls. Daily sperm production/g was similar among breeds, and the difference on DSP was as a result of smaller testicular size in BA. Spermatogenic apoptosis was not affected by breed type.

A third group of 7 Brahman (Brahman, n=2 and miniature Brahman, n=7) bulls as well as 6 Romosinuano bulls were evaluated for testicular traits, spermatogenic traits (DSP and DSPG, using a TD=5.11 for Brahman and miniature Brahman and a TD=5.32 for Romosinuano), and spermatogenic apoptosis. This information was of interest because there is little information on reproduction traits for these breeds. Paired testicular volume, paired testicular weight, paired epididymal weight, and DSP were smaller in miniature Brahman than in Brahman bulls ($P \leq 0.05$). In contrast, testicular circumference, DSPG, and spermatogenic apoptosis did not differ among breeds.

Romosinuano bulls in experiment 3 were 67 d younger than Romosinuano bulls in experiment 1. Romosinuano bulls in experiment 3 showed similar testicular traits (paired testicular volume, paired testicular weight, paired epididymal weight, and testicular circumference) when compared with Romosinuano bulls in experiment 1, although DSP and DSPG were less in Romosinuano in experiment 3 than for Romosinuano bulls in experiment 1.

In Chapter 5, when testicular traits and extragonadal sperm reserves (ESR) were assessed during the Florida summer in B. taurus and B. indicus bulls, An bulls were heavier and some testicular traits were greater when compared to Br, Ro, and NB. However, spermatogenesis and epididymal sperm reserves were not different among breeds, with the exception of ESR (corpus) in Br bulls. When the crossbred breeds used in the present study (BA, SA, and TA) were compared during the florida summer, testicular traits and spermatogenic apoptosis did not differ among breed types. In contrast, DSP and perhaps DSPG, were found to be lower in BA bulls when both were calculated using a TD for B. taurus. In contrast, when a TD for B. indicus was used for BA bulls only, DSP was lower ($P \leq 0.05$) in BA bulls compare to SA and TA bulls. Also, more information regarding testicular traits, spermatogenesis, and spermatogenic apoptosis was obtained for miniature Brahman as compared with Brahman bulls, as well as for the tropically adapted Romosinuano (B. taurus) bulls during the summer in Florida. Brahman and miniature Brahman bulls were considerably older than the rest of bulls used in this series of experiments, and the levels of spermatogenic apoptosis in these bulls were considerably higher than the levels observed in younger animals in this study, suggesting that age could influence the levels of spermatogenic apoptosis.

In conclusion, experiments in this study failed to observe increases in spermatogenic apoptosis in the bull as a result of E. coli endotoxin infusion, scrotal insulation (48 h), gossypol toxicity, or summer temperatures in central Florida. Also, breed type did not appear to influence the levels of spermatogenic apoptosis when tropically adapted and temperate breeds were compared. The reasons for these findings

could include 1) inappropriate detection windows used in experiments in Chapter 3, 2) none of the treatments used in this series of studies caused an increased in spermatogenic apoptosis, 3) germ cell death resulting from elevated temperatures or gossypol toxicity might take a different form of cell death than spermatogenic apoptosis, 4) an age influence on levels of spermatogenic apoptosis, resulting in higher levels in older than in younger bulls, making detection of small changes in spermatogenic apoptosis in young bulls difficult.

Gossypol increased sperm abnormalities and decreased spermatogenic traits (DSP and DSPG), but gossypol showed no effect on spermatogenic apoptosis. The antioxidant vitamin E fed at 4,000 IU vitamin E/bull/d counteracted the spermatoxic effect of gossypol in all parameters evaluated in this study, suggesting that vitamin E supplementation when feeding cottonseed meal could allow the safe use of this valuable feedstuff with intact bulls.

Overall this study failed to demonstrate differences in semen and testicular traits among crossbred or B. taurus and B. indicus (tropically adapted vs temperate) bulls compared in Chapter 5. Spermatogenic efficiency per gram of testicular parenchyma (DSPG) was lower in BA B. indicus x B. taurus crossbred bulls (Chapter 5, experiment 1) when a TD for B. taurus was used to calculate DSPG for BA only. In contrast, if a TD for B. indicus was used DSPG did not differ.

This series of studies suggests that future work should be oriented to identify an optimal time to measure spermatogenic apoptosis after a heat insult such as scrotal insulation for 48 h. These experiments should include a positive control group where

scrotal insulated (48 h) bulls should be kept and ejaculated every 3 days to ensure that the scrotal insulation treatment causes the spermatogenic damage that is expected. Also, semen should be collected in all bulls to measure semen markers for spermatogenic apoptosis such as Hsp70 level. In this experiment, spermatogenic apoptosis should be assessed (by histology, by tunel stain, by DNA laddering, and by cell detection ELISA, as well as by new technologies) daily until d 16 after initiation of scrotal insulation (0 d). Spermatogenic apoptosis results should be correlated with sperm morphology obtained in the positive control group, as well as with semen markers such as levels of Hsp70 in bull semen.

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BIOGRAPHICAL SKETCH

Victor Hugo Monterroso Pérez was born October 1, 1965, in Guatemala City, Guatemala. He is the third son of Nehemias Monterroso Salvatierra and Zoila Lidia Pérez Mazariegos. He was raised in Guatemala City and in the town of San Pedro Yepocapa. He received his bachelor's diploma in sciences from the "Adolfo V. Hall" Military Institute of the Guatemalan Army in 1982, and later he was commissioned as an Infantry Lieutenant. Victor Monterroso was subsequently admitted to the College of Veterinary Medicine at the University of San Carlos of Guatemala. He finished his course work in 1989 and in the same year married his wife, Norma I. Mora, and moved to Miami, Florida. In Spring 1991, Victor enrolled at University of Florida as an undergraduate student of the Dairy Science Department where he had the opportunity to work under the supervision of Dr. Peter J. Hansen. The journal article "Regulation of bovine and ovine lymphocyte proliferation by progesterone: modulation by steroid receptor antagonist and physiological status" published in Acta Endocrinologica (1993) was a product of his undergraduate research. In December 1992, he received his Bachelor of Science degree from the University of Florida, and in Spring 1993 he enrolled as a graduate student in the Department of Dairy Science. In March 11 of the same year his son Victor Alejandro was born, making him a blessed father. In August 1995, he received his Master of Science degree from the University of Florida.

Upon completion of the Master of Science degree in 1995, he completed all the requirements to obtained the title of Medico Veterinario at the degree of Licenciado (veterinarian) from the University of San Carlos of Guatemala in his native country of Guatemala. At the same time he began his doctoral degree in Veterinary Medical Sciences at the College of Veterinary Medicine of the University of Florida where he is now a candidate for the degree of Doctor of Philosophy. In 1997, he was awarded the Florida Veterinary Medical Association Auxiliary Graduate Studies Scholarship. In that year, he became the President of the Veterinary Graduate Student Association and the college representative of the University of Florida Graduate Student Council. In 1998, he was awarded the Auxiliary Graduate Studies Scholarship. After completion of his studies, he is planning to pursue either a training in laboratory animal medicine, a training in food animal medicine, a reproductive physiology postdoctoral position, or return to his native Guatemala.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Peter J. Chenoweth, Chair
Associate Professor of
Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Louis F. Archbald
Professor of Veterinary
Medicine

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Claus D. Buergelt
Professor of Veterinary
Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Assistant Professor of
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Veterinary Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1998

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